VOLUME 10 May 1961 NUMBER 3

THE AMERICAN JOURNAL OF

Tropical Medicine and Hygiene

Combining The American Journal of Tropical Medicine and The Journal of the National Malaria Society

OFFICIAL ORGAN OF
THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

PUBLISHED BIMONTHLY BY
THE WILLIAMS & WILKINS COMPANY
BALTIMORE 2, MARYLAND, U.S.A.

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Correspondence concerning business matters, subscriptions and orders for reprints should be addressed to The Williams & Wilkins Company, 428 East Preston Street, Baltimore 2, Maryland, U. S. A.

Manuscripts and books for review should be sent to Dr. Paul C. Beaver, 1430 Tulane Avenue, New Orleans 12, Louisiana.

The subscription price is $10.00 per year in U.S.A.; $10.50 in Canada; $11.25 elsewhere. Single copies, when available, $2.50 in U.S.A.; $2.75 elsewhere.

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Vol. 10 *May 1961* *No. 3*

CONTENTS

The worms: can we vaccinate against them? **Norman R. Stoll** 293
Presentation of the Walter Reed Medal for 1960 to Sir Gordon Covell 304
Presentation of the Joseph Augustin LePrince Award for 1960 to Dr. Justin M. Andrews 308
Simian malaria in man. **Henry K. Beye, Morton E. Getz, G. Robert Coatney, Harvey A. Elder and Don E. Eyles** 311
Chloroquine resistance in *Plasmodium falciparum*. **Martin D. Young and Donald V. Moore** 317
A possible mechanism of prolonged antimalarial activity. **Leo E. Gaudette and G. Robert Coatney** 321
Seven years' malaria research and residual house spraying in Netherlands New Guinea. **D. Metselaar** 327
Laboratory studies on 3-chloro-9-(4-diethylamino-1-methylbutylamino) acridine, 10-oxide dihydrochloride (CI-423) as an antimalarial compound. **Paul E. Thompson, Jack E. Meisenhelder, Haig H. Najarian and Anita Bayles** 335
Human isosporosis in the Western Hemisphere. **Ernest Carroll Faust, Luis E. Giraldo, Gladys Caicedo and Rafaél Bonfante** 343
Serologic diagnosis of *Schistosoma mansoni* infections. III. Isolation and purification of antigen from adult *S. mansoni* for the complement fixation test. **Saul Rieber, Robert I. Anderson and Myron G. Radke** 351
Splenic reactions in Swiss albino mice to single and multiple infections with *Schistosoma mansoni*. **A. Magalhães Filho and E. Coutinho-Abath** 356
The effect of TWSb on the electrocardiogram of children suffering from urinary bilharziasis. **S. Awwaad, M. Attia and M. Reda** 365
Demonstrated control of *Australorbis glabratus* by *Marisa cornuarietis* under field conditions in Puerto Rico. **Myron G. Radke, Lawrence S. Ritchie and Frederick F. Ferguson** 370
Epidemiology of echinococcosis in the Middle East. I. Human infection in Lebanon, 1949 to 1959. **Calvin W. Schwabe and Kamal Abou Daoud** 374
Side effects of Ciba-1906. **Leon Goldman** 382
Ndumu virus, a hitherto unknown agent, isolated from culicine mosquitoes collected in northern Natal, Union of South Africa. **R. H. Kokernot, B. M. McIntosh and C. Brooke Worth** 383
St. Louis encephalitis in Panama. II. Survey of human blood for antibodies against St. Louis and two related group B viruses, Ilhéus and yellow fever. **Enid de Rodaniche and Carl M. Johnson** 387
St. Louis encephalitis in Panama. III. Investigation of local mammals and birds as possible reservoir hosts. **Enid de Rodaniche and Pedro Galindo** 390
Isolation of the virus of Ilhéus encephalitis from mosquitoes captured in Panama. **Enid de Rodaniche and Pedro Galindo** 393
Birds as hosts of Ilhéus encephalitis virus in Panama. **Pedro Galindo and Enid de Rodaniche** 395
Virus isolations from forest mosquitoes in San Vicente de Chucurí, Colombia. **Hernando Groot, Alberto Morales and Hernando Vidales** 397
The virulence of West Nile virus and TP 21 virus and their application to a group B arbor virus vaccine. **Winston H. Price, Ralph W. Lee, III, William F. Gunkel and Walter O'Leary** 403
An acid-fast pathogen of fresh-water snails. **Edward H. Michelson** 423
Field survey of the health of Bachiga children in the Kayonza district of Kigezi, Uganda. **D. B. Jelliffe, F. J. Bennett, C. E. Stroud, M. E. Novotny, H. A. Karrach, L. K. Musoke and E. F. P. Jelliffe** 435
Correspondence 446
Books Received 446
Book Reviews: *Human Toxoplasmosis*, J. Chr. Siim, Editor; *Handbook of Microbiology*, by Morris B. Jacobs and Maurice J. Gerstein; *Wärmestauung und Schwülekrankheiten. Entstehung, Verlauf, Behandlung und Verhütung für Ärzte und Hygienetechniker*, by J. Grober; *Atlas of Medical Mycology*, by Emma Sadler Moss and Albert Louis McQuown; *Infectious Diseases of Children*, by Saul Krugman and Robert Ward 447

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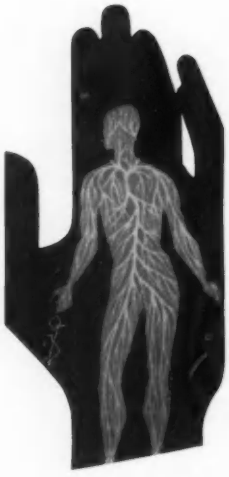

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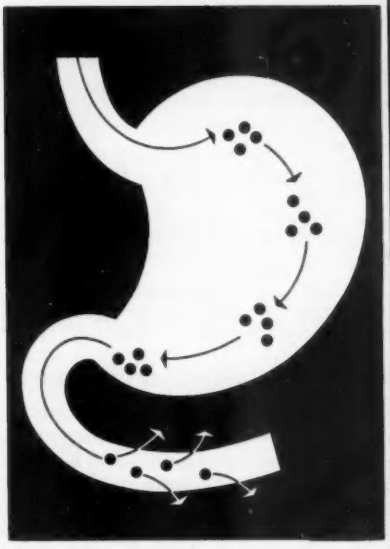

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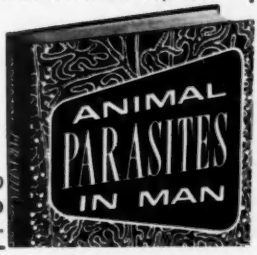

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61034

THE WORMS: CAN WE VACCINATE AGAINST THEM?*

NORMAN R. STOLL†

The honor of presenting the 25th Charles Franklin Craig Lecture is enhanced for me in two ways. For one, my subject, on account of Col. Craig's own concern with the immunological aspects of parasitic infections, would, I like to think, have had an unusual interest for him in depicting one of the directions in which worm-host relationships are now being so actively studied. For the other, it allows the list of topics of lectures given from the Craig rostrum to include another dealing with the helminths. The most recent was the 18th at Louisville in 1953 by Dr. H. E. Meleney[1] on "Problems in the Control of Schistosomiasis." Before that in the 9th at St. Louis in 1944, Dr. L. T. Coggeshall[2] devoted half his lecture to "Filariasis in the Returning Service Man." The 4th Craig Lecture in Memphis in 1939 by Dr. W. H. Taliaferro[3] was on "The Mechanism of Acquired Immunity to Metazoan Parasites." This last touches closely my present subject, and for those interested, it has the virtue of still being recommended reading.

To be aware of the recent successful field trials abroad of vaccination against cattle lung worm is to realize that the question of my title is a rhetorical one. It was, even before that demonstration. And to learn of request for federal licensing in the United States to sell vaccines for three different worm-host systems in the domestic animal field suggests that similar active interest for helminthic problems in tropical medicine cannot be far off.

Not that suddenly the way is made straight, and the conquest of worms in domestic animals and man is just around the corner. Rather that there is help in this problem of the helminthiases. The help is from an angle we have been slow to assign as a resource utilized by hosts in their protection against such parasites, namely, acquired immunity. Nor need we, as in the early history of bacteriology, go off the deep end and assume each parasite-host relationship troubling us will yield to a simple vaccination solution. It is rather that the ingredients of successful approach may be present with a certain number and variety of forms. The Glasgow group,[4] for instance, has mentioned exploring attenuated vaccines involving *Fasciola hepatica*, *F. gigantica*, *Cysticercus bovis*, *Ascaris lumbricoides*, *Metastrongylus apri*, *Dictyocaulus filaria* (as well as *viviparus*), *Haemonchus contortus*, *Nematodirus*, and hookworms. Some of these are familiar names beyond the field of animal husbandry, and those that are not have close taxonomic relatives that are.

One needs to emphasize that the idea that against parasitic worms one may use a vaccine (defined for my use as "an inoculable immunizing agent") rests on a simple thesis. It is that hosts of such worms are themselves able to develop protection through the biological experience of having been earlier infected or reinfected. In the period roughly since World War II this has become a reasonably orthodox point of view. It was not always thus, and it may be worth pausing to recall a few of the steps that favored the idea, and how slowly it caught on.

Worms are metazoa, and two early non-worm metazoan studies stand out. Four decades ago Reuling[5] demonstrated under experimental conditions in Iowa that the largemouth black bass, *Micropterus salmoides*, acquired a protective immunity after as few as two exposures to glochidia of the fat mucket, *Lampsilis luteola*. Mussels have an early larval stage, the glochidium, that after hatching from the egg requires a brief period embedded in the superficial gill- or fin-tissue of an appropriate fish, before being capable of settling down as an independent organism. Gills on immune fish do not permit this parasitism. Arey[6] confirmed and extended Reuling's findings.

More familiar is the report by Blacklock and Gordon[7] who worked in Sierra Leone some three decades ago with the myiasis-producing tumbu fly. The larvae of *Cordylobia anthropophaga* require residence in the skin of man or animals for about ten days. These workers early found in

* Presented as the 25th Charles Franklin Craig Lecture before the American Society of Tropical Medicine and Hygiene, Los Angeles, California, 2 November 1960.

† The Rockefeller Institute, New York 21, New York.

guinea pig experiments: "Regarding our figures as a whole, the fact which primarily impresses us is that the first experimental infection with larvae yields many more larvae attaining to the sixth day of development than all the subsequent infections taken together."

Blacklock and his colleagues knew not of Reuling's work, that fish acquired an immunity to glochidia; but they were acquainted with, and recognized, the "experimental proof of immunity against metazoan parasites as a result of previous infection" furnished by Fujinami[8] in Japan. Because it ranks historically as the first competent evidence in its field, I wish to quote the abstract made from the original Japanese text by R. G. Mills, and published in the *China Medical Journal* in 1917 under the title "Immunity to Macroparasitic Disease, Can it be Acquired?"

"During the author's study of schistosomiasis in endemic regions he observed several facts that made him doubt the frequency of repeated infections even under exposure. Children were observed to be much more affected than adults, while those of any age who came into the endemic districts for the first time were usually taken sick after their first work in the rice fields. Calves brought in from the outside were seen to sicken soon after and many died. Those that recovered, however, seemed to be more resistant in later years.

"There was a horse kept in the laboratory, designated No. 10, which two years before had been invaded by the parasite but had recovered. The author's previous work on the longevity of the fluke in the horse convinced him that in this animal the worms were all dead. This horse, and two more (Nos. 13 and 14) that had never had the disease, were exposed to identical conditions, i.e. were compelled to stand with their legs in canal water known to be heavily infected with the cercaria. Horse No. 14 was kept there somewhat longer than the other two. In two months this horse died and at autopsy more than 20,000 parasites were taken from the portal system. Microscopically, there were demonstrated numerous areas of granulation tissue in the liver produced by the deposition of the eggs. About one month later the other two animals were killed and examined for parasites. In horse No. 13, which three months before had been exposed to infection for the first time, there were found about 10,000 parasites in the portal system and the liver presented a picture identical with that of No. 14. On the other hand, careful examination of the body of horse No. 10 failed to reveal a single worm. The liver sections of this case presented the picture of healed lesions and scattered clumps of degenerated eggs in almost obliterated foci."

In the nematode field, Ducas,[9] working in Brumpt's laboratory in Paris, reported in 1921 a series of experiments with rats in which an appreciable immunity developed after previous infection with *Trichinella spiralis*. In his survey of the literature he not only refers to the Fujinami results but to an earlier observation of Brumpt[10] (1913), who observed that inasmuch as in Abyssinia, where *T. saginata* is common, individuals exposed to successive infections with the beef tapeworm generally show only a single parasite, "il semble qu'une première infestation donne une immunité temporaire qui disparait quand le ver a été expulsé." Ducas also refers to experiments of Joyeux[11] of rats showing immunity to second infections with *Hymenolepis fraterna*.

Thus did coming events cast their shadows before. There is by now an impressive series of observational and experimental results that tell us a mammal is something more than a warm cave or hiding place for worms. The first that get in may do so without let or hindrance, but later members of the same species, and sometimes of related species, receive less and less hospitality. Eventually they find the host resisting their entrance, and turning them out. We say he has acquired an immunity. The more youthful host is characteristically slower to develop such an antagonism. But the callow learn in time and muster intervention as forcefully as do biologically sophisticated adults.

Certain aspects of worm-host systems in relation to acquired immunity are appropriately kept in mind in the over-all problem we are considering. Worms may come in such numbers over a short span, or in such successive hordes, as not to give any biology of protection a fair chance, even in a mature host. Such worms may be relatively large and muscular, carving tunnels in tissue, draining blood, or blocking passageways. If so, initial preoccupation of the insulted host is to circumvent the damage. In consequence, the protective reaction itself may be sabotaged and not become evident. Then we say the host suffers from this, that, or the other verminosis. The host may have trouble even after he has had a back-

ground of successful experience in dealing with invaders. Independently of the parasites themselves, his capacity to react effectively can be undermined. There is, of course, no such thing as an immunity that cannot somehow be broken through.

All of this does not obscure the fact that our familiar hosts have demonstrated capacity to deal offensively and defensively with their worms. Referring especially to the nematodes, whether they enter through the skin or through the mouth, a variety of effects are observed. Late comers are slowed down, or trapped, or prevented from growing, or from growing at a normal rate, in the self-same places within the body where their predecessors had pretty much their own way. Some worms struggle to delayed maturity with their reproductive capacities depressed; or, in a self-cure reaction, they and their siblings are damaged and lose attachment; if they are in a hollow organ communicating with the outside, as in the gastrointestinal or respiratory system, they are then swept out; or, they and their offspring may die in locations from where they are not readily expelled, and their verminous carcasses become involved in ancillary host reactions, sometimes clinically unfavorable, or even tragic.

One[12] may again emphasize that the favorable result for the host emerges under conditions of *re*infection, and when the successive increments of worms are not in themselves too damaging. This is tied to the fact that parasitic worms do not multiply in the host; the size of the infection depends on additions from the outside. Single infections to elicit response are primarily for the convenience of the experimenter, for the single dose does not follow the usual pattern of experience of host and parasite in relation to one another in nature. That investigator is fortunate who happens to deal with a worm-host system where a single infection appears to represent a fair trial for the host, and a fair trial also for himself.

What is required then is that an opportunity be presented to the host so that his defenses may go into action before he is too seriously discommoded, temporarily or permanently, by the pathogenic capacity of the worms. The host biology moves from a condition of susceptibility, to insusceptibility.

Nowhere has this been better shown that with *Trichostrongylus calcaratus* in its natural host, the rabbit, in some beautiful experiments done by Sarles[13] at the Princeton Laboratory of The Rockefeller Institute nearly thirty years ago.

There is another point that ought to be in mind. We like to visualize a protective immunity as a so-called sterilizing immunity, that is to say, the host gets rid of *all* of the invaders. This, of course, is over-simplified biology concerning any parasite-host relationship. Even in such classic illustrations as yellow fever, where neutralizing antibodies are found in *some* individuals for decades after surviving the disease,[14] the only fact we surely know is that the virus has not been later demonstrated. This is not the same as saying it is *not* present. Fairly recent work[15] on a rickettsial infection supports the idea that, with persistent neutralizing antibodies, the infective agent is not absent but rather is hidden and difficult to reveal, and demonstrable only under exceptional circumstances.

There is a school of thought which believes a locus of infectious agent needs to be present if a protective immunity is to be kept up. Work on immunities against helminths supports this, in that such immunities tend to be better maintained, or, in the presence of challenging reinfection, go into action again more promptly, if, as and when *some* worms are still in situ. Far from feeling let down about the helminthic immunity in a host when an investigator finds worms still in residence, there is another way for him to look at it: namely, that the macroscopic helminth permits demonstration of a persistent infective agent, whereas a more minute etiological form could have escaped detection. We have a right to remember the worms we encounter at treatment or autopsy may not be the *only* guests who came to dinner.

Once capacity of self-protection was recognized, investigators attempted to induce the resistant state without going through the process of exposing an animal to infection itself. With the nematodes of mammals, these being my primary concern in this presentation, the use of dead worm material for this purpose has been approximately unsuccessful. The hypothesis naturally followed that secretory or excretory products from living worms were the substances needed. The precipitates found by Sarles,[16] when working in Taliaferro's laboratory, lent support to this view. He found them forming around the apertures of infective larvae incu-

bated in serum obtained from rats hyperimmunized with *Nippostrongylus muris*.

There are various methods of going about introducing to the host the metabolic products of living worms. One way is possible with non-wandering forms, namely, to put them into an abnormal site. Then the host can absorb something from the living worms, without the worms reaching their proper habitat.

This was demonstrated with *Haemonchus contortus* at Princeton. The first published use of the word "vaccination" in a title in relation to worms may have been in the April 1958 *Rice Institute Pamphlet*, concerning those experiments.[17]

Three facts had come to hand about *H. contortus*. First, it induced a self-cure and protection in sheep under conditions of natural or experimental reinfection; second, unlike *N. muris* or *Ancylostoma caninum*, it did not wander in the body of its host, and parenterally introduced forms did not reach their home in the abomasum; and third, its normal, fecally-grown infective stage, to the numbers of thousands of larvae at a time, could be rendered free of contaminating microorganisms. In one set of reported experiments, such live, non-wandering, bacteria-free, exsheathed, third-stage larvae were emplaced intraperitoneally or subcutaneously in a vaccination procedure. Of ten sheep receiving this vaccine, four later showed almost complete immunity; and five others had enhanced protection under challenge that proved fatal or nearly fatal to three unprotected controls. There was also a promising result in a bovine experiment. Evidence obtained from examining the areas of injection in other sheep employed just for the purpose gave a basis for explaining why this was true. They showed that, of intraperitoneally emplaced larvae, after three days most were still alive with some worms exsheathing at the end of the third stage; after six days a few were still alive; but after twelve days no live larvae were demonstrable. For a few continuous days, therefore, the experimental hosts had been exposed to metabolites of living larvae, including such as are produced at exsheathment.

Another way of going about introducing metabolites of living worms to the host is to first collect such secretions and excretions in vitro. Spindler[18] in the U. S. Bureau of Animal Industry appears to have been the first to try this more than twenty years ago. He used clarified digests of rabbit muscle that had contained *Trichinella* cysts. Administered by mouth to rats, rabbits and guinea pigs, "the test animals were protected to a greater or less extent against experimental intestinal trichinosis," as compared to controls. Sixteen years later Thorson,[19] in Cort's laboratory, found that fluids, in which infective larvae of *N. muris* had been suspended for a day, when injected intraperitoneally into rats, protected these hosts in significant degree against challenge infection with normal larvae. In Larsh's laboratory, working with *T. spiralis*, C. H. Campbell[20] using larvae, and Chipman[21] using adult worms, likewise demonstrated that the protective reaction can be stimulated by such fluids after their injection parenterally.

Soulsby, Sommerville and Stewart,[22] however, bespeak the view that though metabolic products of larvae are of value to the host in developing protection, the fluid released during exsheathment of parasitic larvae is the key material required by the host. Experiments in which exsheathing fluid has been used in the absence of the worms, by which to induce an immune state demonstrated by challenge infections, have not been reported. However, Soulsby and Stewart[23] recently presented evidence to show that in sheep infected with *H. contortus* the main stimulation at self-cure appeared to be derived from substances released by larvae during the third ecdysis.

One is inclined to believe, if vaccination procedures are developed by use of metabolites collected from nematodes, their success will come pari passu with improvements in axenic culture techniques. This would be true whether they turn out to come primarily from the gut or excretory pore, or from exsheathing. When Glaser and Stoll[24] in 1938 reported some degree of success with in vitro culture of *H. contortus*, they expressed the hope it would further "elucidation of the mechanism of immunity against helminths by their hosts." We were wanting at that time to collect consequential amounts of metabolites from fourth-stage larvae, or adults, for artificial immunization. Now, twenty-two years later, another investigator[25] remarks: "The time is not far distant when parenteral vaccines prepared from parasites cultivated *in vitro* will be available as commercial preparations."

Axenic culture of parasitic worms is presently

a fast-growing sector of experimental helminthology. A half-dozen authors have abstracts on the problem in the program of the Los Angeles joint meeting with the American Society of Parasitologists. Such interest promises an opportunity to test out on a larger scale before long this particular approach to vaccination.

There is a third method of preparing the host to protect itself against verminous parasites. That is to first present to the host the same, or similar species, whose capacity to develop in normal fashion is reduced. The requirement remains that this reduced capacity be not lowered too much. Ideally, it is lowered to a point where the parasites are only temporarily able to furnish the desired secretions, and not excessively damage the host while doing so. There may be naturally occurring species or strains of helminths in feral or domestic animals that qualify here. In the program of this Los Angeles meeting there is a report by S. Y. L. and H. F. Hsü[26] concerning the non-human strain of *Schistosoma japonicum* that may be in this category. One is given to wonder also whether individuals who have suffered from swimmer's itch, or larva migrans,[27] due to non-human species or strains of parasites, may not be obtaining some degree of effective immunization against related species that fulfill their life history in man. More examination of the possibilities here seems desirable.

The attenuation of normally pathogenic forms by irradiation has in more recent time been reexamined. It will be recalled the experiments of Tyzzer and Honeij[28] in 1916 get credit as the first to show "the effect of radiation on the development of *Trichinella spiralis*," as a step looking toward radium therapy of bladder schistosomiasis. This latter was an unrealized hope. More than twenty years passed before the adverse effects produced on trichinae in rats and mice by roentgen irradiation were brought to attention by Semrad,[29] and Levin and Evans[30] first used x-irradiated larvae in immunization experiments. Since the war there has been increasing interest in the effects produced by irradiation, from various sources, on helminths, although most of this work has continued to be with *T. spiralis*.

In the use of irradiated larvae for use in vaccination, the most striking results of the moment are those that have been obtained by the concerted attack of a group of investigators of the University of Glasgow Veterinary School. Let me sketch their researches briefly.

In the summer of 1952 Jarrett, McIntyre and Urquhart[31] began a comprehensive inquiry on the pneumonias of cattle in southwest Scotland. They reported recognizing at least nine morphological types, including some "cuffing" and inclusion-body pneumonias, but the major disease was "husk" or "hoose," caused by *Dictyocaulus viviparus*. They set about studying this parasitic bronchitis experimentally. They examined significant details of the nematode's life cycle from the control point of view, such as its capacity to overwinter on pasture and the consequent relation to pasture management. They sought out figures on incidence with respect to geographical distribution, morbidity and mortality, and the effects of the lungworms in relation to the lesions of the disease. They found that clinical symptoms had never been correlated with the morbid changes, nor was much known of the significance of the immunity. These were not parasitologists but veterinarians interested in a disease of cattle.

They were early impressed that an acquired immunity existed in the field as reported by other workers (Wetzel[32] in 1948, Grégoire,[33] and Taylor[34] in 1951), and this was supported by their own observations on farms and in experimental infections. To test its biological validity they undertook (joined by Jennings and Mulligan[35]) an experiment in passive immunity. After injecting five calves intraperitoneally with thrice concentrated serum from hyperimmunized cattle, the experimental calves and five uninjected controls were challenged with 4,000 normal larvae each. When later killed and autopsied the control calves had a total of 5,501 lungworms, whereas those passively immunized had only 262, or less than 5% of the controls. These appear to be even better results than Sarles[36] obtained with *N. muris*. One gets the impression indeed, that comparatively, the *D. viviparus*-calf worm-host system is a rather favorable one biologically for immunization development.

Jarrett and his colleagues went on from this to investigate the possible use of irradiated *D. viviparus* larvae as initial infections with which to protect calves against normal larvae. Their experiments appear to have been encouraging from the beginning. In 1957, they[37] were able to

report not only good results obtained in experiments of laboratory type, but also in field tests. Thus of 1,088 calves on 40 farms with a past history of parasitic bronchitis, one-half of the animals were vaccinated, one-half left as controls. The vaccine appeared to be safe under these conditions with no breakdowns in attenuation. Parasitic bronchitis broke out that season on 6 of these farms with 91 animals at risk; of the controls 27 out of 43, or nearly two-thirds, developed clinical disease, but this occurred with only 3, say 6%, of 48 vaccinates.

In their work since they have (with Sharp[38]) undertaken a test of a double, rather than their earlier single, vaccinating dose of attenuated larvae; they have further explored (with Thomas[39]) the immunity resulting from variable types of experimental infection; they[40] have tried, with partial success, immunization with whole worm vaccine administered intramuscularly with Freund adjuvant; and they have re-examined[41] the irradiation dosage appropriate for effective attenuation.

Further, they[42] have in course tried vaccination of sheep against *H. contortus*, using a single dose of x-irradiated larvae, again with encouraging results. At the McMaster Animal Health Laboratory in Sydney, Australia, experiments of similar type and promise are in progress.[43]

The work presented in 1957 by the Glasgow Veterinary School team at the 75th British Veterinary Association meeting "led to the development of the first commercially produced vaccine for use against a nematode infection." The unique problem of quantity production of this kind of vaccine was reported in extenso by Poynter *et al.*[44] at the 78th B.V.A. meeting, in September, 1960. They noted that "during 1959 many tens of thousands of courses of lungworm vaccine were distributed commercially to veterinary surgeons in the British Isles," so that this approach is now being widely tested.

In an editorial[45] on this sequence of findings *Lancet* has observed: "No dead vaccine or extract so far devised has produced adequate immunity; apparently only a live worm in the body is fully active immunologically. Until recently this appeared to be an insuperable obstacle; but in the past few years it has been partly overcome by investigators whose work may prove to have wide applications. . . . Which species will prove susceptible, and which resistant, only experiment can tell."

I have mentioned that the Glasgow Veterinary School team, in investigating infection with *D. viviparus* (a form taxonomically somewhat close to the hookworms), early determined the significance of the immunity it was reputed to produce. They then experimented in the laboratory with the self-same worm and host species from a variety of points of view, and on an almost wholesale scale. This type of approach, available to research in veterinary medicine, is part of its great opportunity to contribute to areas of human medicine where a comparable question and answer method is, in the nature of things, denied the investigator.

One is tempted, nevertheless, to take another look at that harsh and poorly controlled pandemic of the moist tropics—hookworm infection. On a world scale it is man's most insidious and serious helminthic pathogen. As early as 1940 Stoll had joined with Cort and Otto[46] in drawing attention to the hiatus in our past thinking, of ascribing any active functional role to acquired resistance against hookworm in man. Earlier, in 1925, Sawyer[47] had been impressed by the fact that, in general, hookworm-infected communities tend to reach and maintain a level of equilibrium. They return more or less to a status quo although at different rates, following different kinds of control measures. In the careful theoretical analysis he developed to account for this phenomenon, Sawyer did not, however, include any potential host resistance. Even in so recent a synopsis on "hookworm disease" as that of Carr,[48] last brought up-to-date in 1959, the three pages on "resistance to hookworm infection" are almost exclusively devoted to implications from the comparative experimental studies on *Strongyloides stercoralis*, *A. caninum*, and *N. muris*.

It is not strange that this is true.

There is the striking correlation of degree of anemia with increased worm count (Darling, Barber and Hacker,[49] 1920), or increased egg count (Stoll and Tseng,[50] 1925; Smillie and Augustine,[51] 1926; Carr,[52] 1926). There is also, I think, a subversive effect on us all of the photomicrographs of slices through the attached hookworm in situ, showing the extraordinarily efficient sucking apparatus of this pathogen. Its implications are supported by the demonstrations of one-way blood flow containing uncrenated red cells escaping from the ani of sucking worms (Wells,[53] 1931; Roche and Torres,[54] 1960). Many

of you have doubtless seen the vivid five-minute movie prepared by M. S. Ferguson for the Communicable Disease Center, U. S. Public Health Service, showing *A. caninum* adults in vivo, with simultaneous extrusion of blood from the male, and eggs from the female, even while they are in copula.

These and other observations easily foster the conclusion that hookworms can do pretty much as they wish with their hosts. The epitome of this point of view lies in the fact that the amount of hookworm infection in the hosts was, and is, used as a measure of the degree of their exposure to sources of infection. Could we perhaps turn this around and say that the only thing we know we measure is the residual worm burden that gives no necessary clue to the amount of exposure? This would still emphasize the requirement to deal with heavily infected hosts.

But the above is not the whole of the hookworm story. Deterioration of host resistance, particularly due to malnutrition, and iron loss, are known to assist the parasite in getting the upper hand. The words of Bailey K. Ashford,[55] written in 1934 after more than three decades of close association with the classic hookworm problem in Puerto Rico, come to mind: "For the benefit of intelligent readers, both lay and professional . . . we amply acknowledge the very important place taken by nutritional unbalance in producing the picture recognized all over Puerto Rico as *la anemia*, or, in other words, hookworm disease. But the undeniable fact remains that this was not a nutritional, but a parasitic anemia. . . . How then does the influence of poor diet come in? Thus: parasites plus poorly balanced food, bring fatalities and serious grades of anemia, which would not occur from parasites alone." Rhoads and his colleagues[56] reporting from Puerto Rico, also in 1934, noted: "The observation was repeatedly made that removal of parasites resulted in little improvement of hemoglobin level or of clinical condition of patients, sometimes over periods of as long as 90 days. On the other hand, without removal of parasites, prompt improvement of blood values and of clinical condition invariably resulted from administration of iron salts without change in diet."

Then there are unorthodox reports such as those of Gordon[57] (1925) in Sierra Leone, Cort and his colleagues[58] (1929) in some of the Panama areas, and Fülleborn[59] (1929) in North Corrientes Province, Argentina. In each of these a racial component was suggested as the probable factor to explain the relatively small amount of measurable damage even in high egg count groups. Fülleborn went further. Finding higher egg counts in children than in adults believed to have had similar types of exposure, he "suggests that the reduction of infection in the adults may be caused by 'acquired immunity' in consequence of the high and continuous infection during earlier life."

Fülleborn had earlier mentioned: "While North Corrientes, like Paraguay, may be one of the most heavily hookworm-infected countries of the world, the clinical damage among the population is much less in evidence than that reported from other Necator countries (such as Brazil) having a much lighter infection. This is probably because of the better standard of life, especially the better nutrition, of the Corrientes people (they consume a great deal of meat), which makes them more tolerant to their parasites than in the case with the Brazilian peasants, who, according to Smillie, are badly nourished and much overworked. That malaria, so frequent in many hookworm-infected countries, seems to be practically absent among the population of North Corrientes is doubtless another important factor in their favor."

In present view it would almost seem as if Fülleborn had had under observation just the sort of group in which individual acquired resistance was capable of manifesting itself. This condition, he himself points out, is not always true in endemic hookworm areas. He could have quoted the poet:

> "What's done we partly may compute
> But know not what's resisted."

But this is all only indirect support of the idea that acquired immunity may be a major force in restraining hookworm in man, even though interfered with by intercurrent factors. Only meager evidence beyond certain epidemiological facts is available to support the idea yet. Sheldon and Groover[60] (1942) are among the few who have sought other evidence with Sarles' type precipitates. What they were able to demonstrate in Georgia should be recalled. They found such precipitates forming around hookworm larvae when incubated with serum from 6- to 19-year-old children who had been diagnosed as having light and moderate infections. They also found precipitates forming in serum from some children

who came from hookworm-infected families but who themselves had no demonstrable infections. They found no such precipitates in serum from five Boston children aged 10 to 13 years believed never to have been exposed to hookworm. But their inability to demonstrate such precipitates in serum from Georgia children with heavy or very heavy infections, as they also reported, would be quite in accord with my expectation, indicating interference with the capacity of the hosts to get their resistance mechanism into gear while under heavy assault.

One wonders whether Sarles' type precipitates could have some correlation with eosinophilias. In self-cure and protection reactions in the sheep against *Haemonchus*, there was a striking fact concerning the eosinophilic leukocytes. In animals that were developing resistance, or showing resistance on challenge both in experimental tests and in field studies, a marked eosinophilia was a familiar finding; in contrast, in animals responding badly in a clinical way, or not yet developing resistance, the eosinophils in the circulating blood were so difficult to find as to be considered practically absent. Indeed, the ground rule was, if an infected sheep or lamb had an eosinophilia, all was well; if not, begin to worry. In view of the recognition by other workers as well of heavy eosinophilic infiltration around worms and lesions in resistant animals, the excess that spills over into the circulation at this time may be significant in relation to a state of immunity. A possible correlation of this sort of event with Sarles' type precipitates would appear to deserve examination.

When we leave the problem of the hookworm of man and go to the comparative field, direct evidence on the host-hookworm relation has been abundantly adduced from experiments with the dog. Experimentation in Cort's laboratory over a period of years by McCoy, Foster, Kerr, Otto and others, are familiar to you all, and was in part reviewed by Otto.[61] They have furnished adequate support for the conclusion that resistance against *A. caninum* may be acquired by the process of infection and reinfection. Such experiments have gone further and shown some of the factors, notably nutritional, that affect the host and retard or prevent acquisition of an immunity, or render it labile.

Recently Dow and his colleagues[62] (1959) of the Glasgow group have reported that infection with *Uncinaria stenocephala* produces a degree of resistance to reinfection similar to that for *A. caninum*. They too have gone further, but in another direction. They have demonstrated that 1,000 infective larvae of *U. stenocephala*, after irradiation at 40,000 roentgens, and administration by stomach tube to bitch pups, established only light infections; also, that such irradiated larvae were capable of giving rise to a marked immunity as shown by later challenge with 1,000 normal larvae. The first experiments are obviously preliminary, but they are not without promise of bringing this worm-host system into the vaccination area.

And now a few closing words on the over-all problem.

In emphasizing something of the what and how in this report, there are many areas of interest which have of necessity failed of discussion, even of mention. The basic thesis upon which any vaccination against worms is to be postulated might have had more illustrations from diverse forms, both parasite and host. For instance, the significant observations on cestodes, both larval and strobilate, have not been detailed; nor has the work on nematodes in the chicken. Also almost nothing on serology has been included; and the words "antigen" and "antibody," it will have been noticed, have not been used. I can only ask the indulgence of those many investigators whose work has influenced the development of our thinking in this field, but whose names have not found place in this brief report. References to many of them have been given in another place.[17]

A major omission has to do with the question of, "Why does the immunization work?" On this score perhaps Dr. Taliaferro will consider bringing his 4th Craig Lecture[3] on "The Mechanism of Acquired Immunity to Metazoan Parasites" up-to-date and catch points raised in the last twenty-one years, especially the recent ones, and including those made by the Australian workers.

It might be mentioned, however, that, in a cognate area, the exact mechanism of protection was not known at the time cowpox vaccination became a useful method for the public health (and I believe this is not yet known).

At the Rice Institute Symposium nearly three years ago, the present speaker[17] said: "In the general scheme of things vaccination against worms could conceivably make a place for itself, as have immunities acquired through the normal process of infection and reinfection, and as have nutrition, and anthelmintics, and genetics, although none of these, in present view, can *alone*

take over control of worm-host systems, for the total protection of the host." As concerns the worms of man we are not apt to fail to realize this. Nevertheless the dawn of the vaccination era against helminths will hereafter put us on the qui vive on one score. In future studies on the epidemiology of human helminthiases, we will be evaluating more consciously whether active immunity can be assigned a place.

For such evaluation we are handicapped, unfortunately, in not having serological methods available to determine whether a host *is* protectively immunized against its worms. The only sure way at present is the result of exposure to new infection itself. With the viruses there is the useful serum neutralization test. We have one guide in Sarles' precipitates which have recently been restudied by Jackson[63, 64] with the fluorescent technique. There may be other useful methods to determine a state of active immunity against helminths. As mentioned, hypereosinophilia needs reexamination from this angle. Would perhaps these "other useful methods" come sooner, if we could encourage the channeling of some of the perennially exuberant research interest[65] in *Trichinella* (in view of its rather specialized character as a helminthiasis), and steer more investigation toward forms taxonomically closer to those that constitute our major problems?

Awareness of the vaccination potential against worms properly allows raising another point. Someone has said that the discovery of antibiotics set back chemotherapy twenty years. Now one may predict a period of avid exploration of helminthic vaccines. It would be unfortunate if such preoccupation should depress and discourage the resurgence of interest and accomplishment that has been true since World War II in the development of more and better anthelminthics. As emphasized, no one aspect of control of the worms of man, including even nutrition, will furnish the complete answer. It will take much energy, and on many fronts, before we approximate the goal of a worm-free human society. In the meantime it is fair to assume we are developing new means to help us toward it.

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PRESENTATION OF THE WALTER REED MEDAL FOR 1960 TO SIR GORDON COVELL

At the annual meeting of the American Society of Tropical Medicine and Hygiene, held at Los Angeles, California, on Friday, November 4, 1960, in the presentation of the Walter Reed Medal *in absentia* to Sir Gordon Covell, **Dr. Justin M. Andrews,*** Member of the Walter Reed Medal Committee, said:

Mr. President, members, and guests of the American Society of Tropical Medicine and Hygiene:

The bestowal, at various appropriate intervals, of the Walter Reed Medal upon individuals widely known for their original researches in some aspect of tropical medicine was authorized by this Society in 1936. The Awards Committee was specified to consist of three past presidents of the Society. The present Committee is comprised of Dr. William Hays Taliaferro, Chairman; Dr. Frederick Brady; and me. Regrettably, neither of the other two members could be present at this meeting, so I am left with the pleasant responsibility of announcing our current choice of many potential candidates, and of presenting this award to him.

After several exchanges of opinion by correspondence, the Committee unanimously selected Sir Gordon Covell as the recipient of the honor for this year. A somewhat honorific biography of him has been prepared—and reads as follows:

Major General Sir Gordon Covell was born on October 20, 1887, somewhere in England, son of Edwin Louis Covell. He was educated in King's School in Canterbury, and in 1913 earned his baccalaureate and medical degrees from Guy's Hospital and Medical School. Nine years later he was awarded a Diploma in Tropical Medicine and Hygiene by the London School of Hygiene and Tropical Medicine. In 1923 he received a doctoral degree in medicine from the University of London, and in 1929 another in public health.

Sir Gordon served most of his professional career in India as a commissioned officer in the Indian Medical Service, in which he rose to the rank of Major General. India had developed into a fertile field for tropical medicine research through the findings of Ross, Leishman, Donovan, Patton, and others. Malaria was the scourge of the country. It occurred under such a perplexing variety of conditions that the Royal Society sent a Malaria Commission to India in 1900 to study the situation. Its report led to the foundation in 1909 of a Central Malaria Bureau at Kasauli, under the direction of Sir Rickard Christophers, for research, training, and the dissemination of knowledge about malaria. This involved, as I understand it, a Central Committee and many Provincial Committees, members of which met periodically to discuss the malaria situation—and a place where Sir Rickard could store and study anophelines.

After the first World War, it became apparent that a more adequate, permanent antimalaria organization was needed, and in 1927 this was proposed by Christophers and established in Delhi under the designation, Malaria Survey of India, with functions similar to but broader than those of the Central Bureau. At this point, Sir Gordon, then a Major in the I.M.S., entered the scene as Assistant Director to Major J. A. Sinton, I.M.S., the first director of the organization which at that time was not governmental but was supported by the Indian Research Fund Association.

A remarkable and productive group was assembled by Sinton. In the words of Covell, who reviewed its history some years later, the Malaria Survey of India owed its inception to Sir Rickard Christophers, but the reputation it earned as a research organization, chiefly to the able and energetic direction of Sinton.

The names of many of its workers are familiar to all students of malariology and entomology—Captain P. J. Barraud, I.M.S., who worked on the culicine fauna of India; Dr. Bruce-Mayne, who later established the U. S. Public Health Service Malaria Laboratory in Columbia, South Carolina; Mr. R. A. Senior-White, who became the malariologist to the Bengal-Nagpur Railway; and Lt. Col. T. C. McCombie Young, Major R. C. Watts, Major R. W. Milligan, and Major

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M. K. Afridi, all of the Indian Medical Service. Dr. George Macdonald, currently Director of the Ross Institute, an adjunct of the London School of Hygiene and Tropical Medicine, was research officer from 1929 to 1931. There were many others, including Drs. Marshall Barber and Justus Rice. Most of the researches of this group were published, beginning in 1929, in the *Records of the Malaria Survey of India.*

In 1934, Sir Gordon was appointed Director of the Pasteur Institute of India, but returned in 1936 to succeed Sinton as Director of the Malaria Survey. In late 1937, the Government of India assumed support of the organization which was known thereafter as the Malaria Institute of India, and the previously published *Records of the Malaria Survey* became the *Journal of the Malaria Institute of India;* in 1947, the same series of publications was recommenced as the *Indian Journal of Malariology.* All of these were edited by the director of the Institute.

Sir Gordon retained his position as Director of the Institute until 1947. During World War II, he advised importantly regarding the British program of malaria control in the various theaters of military operations, and directed much of the antimalarial activity in and around military and Royal Air Force installations in India. Staff for the first two British malaria control units was supplied by the Institute, and personnel were trained there for 100 other such units.

In spite of heavy administrative responsibilities and much local malaria survey activities carried on while in India, Sir Gordon authored or co-authored some 31 original articles and several books. These reveal the extraordinary variety and depth of his malariologic interests. They include such considerations as malaria survey techniques; the malaria transmission potential of various anopheline species; the distribution and ecology of anopheline species in India and the Far East; the comparative anatomy of different species both as adults and as larvae; keys to anopheline species; the relation of gametocyte density to mosquito infectibility; splenometric techniques and the significance of splenomegaly vs. malaria endemicity; the vital statistics and epidemiology of malaria; man-made malaria; seasonal outbreaks of malaria; the physical effects of malaria on man, especially under military circumstances; pyrethrum control of adult anophelines; mosquito traps and preferred anopheline resting places in nature; the comparative effectiveness of various larvicides; the chemotherapy of malaria; and many others. Indeed, it is hard to think of any aspect of malariology which has not been explored and discussed by Sir Gordon, especially within the framework of the tremendously diverse demographic, climatic, and topographic conditions of India.

His last paper written before leaving India is an amusing report entitled, "Bats, Clover, Water Lettuce, and Malaria," in which he casts grave doubts on the allegations of previous authors concerning the ecologic relationships of these variables from the standpoint of malaria control.

He is the senior author of "Chemotherapy of Malaria" (1955) a WHO Monograph written in collaboration with Dr. G. Robert Coatney, Dr. Jaswant Singh, I.M.S., retired, and Dr. John W. Field.

While in India (1939), Sir Gordon was made a Companion of the Indian Empire (CIE). In 1946, he was created a Knight and was Honorary Physician to the King (KHP) from 1944 to 1947.

His outstanding contributions as a teacher, adviser, medical scientist, and director of laboratory and field research in malariology and malaria control have touched the lives not only of his immediate associates but of practicing malariologists the world over. In an even larger sense, the fruits of his labors have prevented illness, prolonged life, and made existence more endurable for literally millions of persons.

It is for these considerations that the Awards Committee has recommended and the Society Council has approved that the Walter Reed Medal be bestowed this year upon Major General Sir Gordon Covell.

We regret immensely that he is unable to be here to receive it in person. In his unavoidable absence, we are transmitting the Medal to him through the offices of his close friend and admirer, Dr. G. Robert Coatney, who assures us that he will see to it that this evidence of our high esteem is delivered to Sir Gordon.

At the ordinary meeting of the Royal Society of Tropical Medicine and Hygiene,* held at Manson House, 26 Portland Place, London, W.1, on Thursday, December 8, 1960, in the presentation of the Walter Reed Medal, **Professor George Macdonald** said:

* *Tr. Roy. Soc. Trop. Med. & Hyg.*, **55**: 15–16.

The American Society of Tropical Medicine and Hygiene has awarded the Walter Reed Medal to Sir Gordon Covell. This is one of the two awards with which the Society honours research workers in the field of tropical medicine, the other being the Le Prince Medal given in the field of malariology, and it has a standard in the American Society ranking with that of the Manson Medal in our own—the highest indication of respect within its power. The announcement of the award was made at the Society's Annual Meeting at Los Angeles in November, 1960, and that Society greatly regretted that Sir Gordon was unable to be present to receive it in person. The suggestion that the presentation might be made by our President on behalf of the American Society at one of our meetings was very warmly received, and I have in consequence the privilege of giving you this message.

The announcement was made by Dr. Justin Andrews on behalf of a Committee of Award consisting of three Past Presidents. His speech reviewed Sir Gordon's professional career from his first joining the Indian Medical Service in 1914 through 46 years of creative work, almost entirely since 1918 in the field of malaria. The problems of malaria as he found them were intriguing, stimulating, and a worthy challenge to the intellect. He responded to the challenge by research continued to the present day, which has been immensely fruitful, and he has supplemented that research by great activity and resource in the fields of education, training and administration. He has thereby enriched the whole world of malariology, and in India there is no aspect of the present vast research and operational work on malaria which does not owe much of its foundations to him.

I know from the terms of the announcement, and from the reaction of the Members of the American Society of Tropical Medicine and Hygiene on hearing it, that the award brings with it the warmest and most sincere appreciation of that Society.

The happy arrangement that our President should hand the Medal on behalf of that Society to Sir Gordon allows us the unique opportunity of sharing the American Society's pleasure in giving, and Sir Gordon's in receiving. I have had the privilege of conveying this message and the Medal from America, and would like to add my own respects and salutations to our distinguished Fellow who has brought learning and life to every organization with which he has been connected, including this Society. I ask, Sir, that you should present the Walter Reed Medal and its accompanying Certificate to Sir Gordon Covell on behalf of the American Society of Tropical Medicine and Hygiene.

The President, Sir William MacArthur, then presented the Walter Reed Medal and Certificate to **Sir Gordon Covell** who said:

I wish to express my deep appreciation to the American Society of Tropical Medicine for conferring on me the Walter Reed Medal; and to you, Sir, for consenting to make the presentation on its behalf.

I would also like to thank Professor Macdonald, with whom I have been closely associated since he came out to India in 1927, for his very kind remarks.

The name of Walter Reed is one of those most honoured in the field of tropical medicine. The demonstration by him and his devoted band of helpers of the mosquito transmission of yellow fever, work which could only be done with the aid of human volunteers, led directly to the organization of effective measures of control. Reed's experiment was done in 1900, the year in which Manson confirmed the mosquito transmission of malaria.

In the following year Gorgas freed Havana of the scourge of yellow fever within the space of three months by an intensive attack on the mosquito vector. Later, by similar methods, he made possible the construction of the Panama Canal and rendered it safe for the passage of the world's commerce.

In January 1914 I was present at a dinner given to Gorgas at the Royal Army Medical College in London. I did not know him personally, but in after years I had a number of discussions with Joseph LePrince, who was his right hand man both in Cuba and in Panama.

Those of you who attended the 4th Congress on Tropical Medicine held in Washington in 1948 may remember that, at a meeting held to commemorate the work of Walter Reed, one of his colleagues, James Hanberry, was present on the platform. Hanberry was one of the volunteers who for 20 days slept in a mosquito-proof room on beds covered with the soiled and foul-smelling clothes of yellow fever patients, in order to explode the notion that fomites could carry the infection.

Subsequently, he and several others were

successfully inoculated either by the bites of mosquitoes that had fed on yellow fever patients, or by the injection of infected blood or serum.

The hazardous nature of this experiment may be judged from the fact that the mortality from yellow fever in an epidemic at this period was often as high as 40 per cent.

May I say in conclusion that I am particularly glad to receive this honour because during the past 30 years I have had a number of personal contacts with seven of the ten previous recipients of the medal, namely, Herbert Clark, Rolla Dyer, Lewis Hackett, Paul Russell, Steve Simmons, N. H. Swellengrebel and Willard Wright.

I am very proud to be thought worthy of inclusion among such a distinguished company.

PRESENTATION OF THE JOSEPH AUGUSTIN LePRINCE AWARD FOR 1960 TO DR. JUSTIN M. ANDREWS

At the annual meeting of the American Society of Tropical Medicine and Hygiene, held at Los Angeles, California, on Friday, November 4, 1960, in the presentation of the Joseph Augustin LePrince Award, **Mr. Lawrence B. Hall*** said:

Mr. President, members, and guests of the American Society of Tropical Medicine and Hygiene:

The Joseph Augustin LePrince Award has become an established part of the meetings of the American Society of Tropical Medicine and Hygiene, but not all of the present membership had the opportunity of knowing the outstanding engineer, scientist, and great American in whose name this award was established. For those of us who had that opportunity, there will always remain the mental image of the tall, lanky figure, of the quick smile, of the simple and direct approach to complex problems. It was, perhaps, his ability to simplify problems that led Joseph Augustin LePrince through forty years of service to mankind in ridding areas of the world of yellow fever and malaria.

It was in Cuba, in 1901, that he first joined forces with General Gorgas to demonstrate to the world that the pest hole that was Havana in those days could be made a safe place to live. He followed this success by similar work in the construction of the Panama Canal and by control of malaria in extra-cantonment areas during World War I. The latter years of his career were devoted to improving the tools and techniques available for malaria control in those days and to demonstrating methods of malaria education for school children, an effort in which his simple, direct approach achieved marked success.

I am sure, having had the privilege of knowing Mr. LePrince and his sense of values, that he would most fully approve of the action of the Council of the Society in bestowing the award for 1960 on Dr. Justin M. Andrews, Past President of our Society and Director of the National Institute of Allergy and Infectious Diseases.

* Technical Development Laboratories, Technology Branch, Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Savannah, Ga.

Dr. Andrews began his active work in malaria control, following some years on The Johns Hopkins faculty, as Director of the Division of Malaria and Hookworm Service of the Georgia Department of Public Health. From 1938 through 1942, he established in Georgia a program that developed many of the procedures and techniques that were used in the campaign to eradicate malaria from the United States. During the war years, Dr. Andrews served the United States Army in malaria control in Africa and the Pacific. He was awarded the Legion of Merit for contribution to malaria control in North Africa and Italy. In 1946 he was appointed Deputy Officer in Charge and in 1952 Officer in Charge of the Communicable Disease Center, in which capacity he served until 1953 when he was appointed as Assistant Surgeon General and Associate Chief of the Bureau of State Services of the Public Health Service.

It was during Dr. Andrews' tour of duty as Chief of the Communicable Disease Center that he directed the multi-disciplined campaign of that organization and the several states to eradicate malaria from this country. The magnitude of the project, complicated by the return of many carriers in our Armed Forces, made its attainment seem as fanciful as were dreams of space ships in that day; but the continuing absence of malaria from the boundaries of our country attest to the soundness of the planning of Dr. Andrews and the other leaders of the eradication effort. The contribution of Dr. Andrews' leadership and foresight in this era simplified the selection of this year's recipient of the Joseph Augustin LePrince Award.

The award this year has been made possible by the H. D. Hudson Company, a firm which, in the best traditions of United States industry, has provided many of the tools of malaria eradication.

Dr. Andrews, it gives me deep, personal pleasure to bestow upon you, in the name of the American Society of Tropical Medicine and Hygiene, the fourth Joseph Augustin LePrince Award for your outstanding contributions to the eradication of malaria in the United States.

Dr. Andrews' remarks on receiving the award were:

Mr. Toastmaster, Mr. President, fellow members, and guests of the American Society of Tropical Medicine and Hygiene, and Mr. Spokesman for the LePrince Award:

This is by all odds the most eloquent—as well as the most unmerited—encomium that I have ever heard. But even if credibility was strained a little here and there, it leaves me feeling warm and comfortable, and deeply appreciative of the high honor done me this evening, and bestowed by you, Mr. Spokesman, so elegantly. My thanks are due, also, to the Society and to its Committee on Awards—and last, but by no means least, to the H. D. Hudson Manufacturing Company of Chicago, whose products for the application of insecticidal and other sprays are well and favorably known the world over. I often wonder where we would be—and by we I mean the fraternity of science in its most comprehensive sense, and the professional scientific societies in particular—without the assistance and co-operation of our industrial confreres.

I should like to say, for the record, that I have had a long, pleasant, and profitable association with the LePrince Award spokesman for the Society, Mr. Larry Hall, and so I am very happy that he was drafted to make this presentation. Our first contacts were in 1938 or thereabouts, shortly after I had made the hard decision to leave the academic life and had gone to Georgia as its State Malariologist. Mr. Hall, also a Yankee expatriate, hailed from North Dakota. He was a young and aspiring engineer in the Georgia Department of Public Health. In addition to his professional responsibilities, he had a strong interest in photography, especially cinematography, applied to public health education. So, we collaborated with Jack Henderson and Rock Howard, whom many of you know, in producing a promotional movie on malaria control which was exhibited, I suppose, until it fell to pieces.

When I returned to Georgia from duty overseas in World War II, I found that Mr. Hall had been commissioned in the U. S. Public Health Service and assigned to the Malaria Control in War Areas organization, which has since evolved into the Communicable Disease Center. At that time, Mr. Hall was one of the very few mechanical engineers in the Public Health Service, a fact which doubtless is significant with reference to the continuous stream of mechanical inventions and improvements which has emanated from the Savannah laboratories of CDC.

I should like also to make a few remarks concerning the individual for whom this Award was named. I never knew Mr. LePrince intimately as did Dr. L. L. Williams, Jr., his supervisor in the Public Health Service for several years, or Drs. Carter and Griffitts and Mr. Johnson with whom he worked. But I have long felt that he deserved credit not only for his observations on the flight range of anophelines in this country and for the development—with others—of concrete ditch-linings but, perhaps even more importantly, for the practice which he instituted in the Panama Canal construction days of having boys hand-kill each morning the anophelines which had entered the railway cars the previous night to feed upon the laborers quartered in these mobile barracks. These were the mosquitoes which, statistically, were most likely to be concerned with malaria transmission in this population. Their systematic destruction must have had the same drastic reductive influence upon malaria incidence as that exercised on a much broader scale almost a half century later by space and residual insecticides. I suspect that the principle was the same in both instances, though the actual practices were clearly different.

I suppose I first saw Mr. LePrince in the early 30's, as it was about this time that I started to attend the meetings of the National Malaria Society, of which he had been one of the founding fathers in 1917. He visited me in Georgia in 1941 or 1942. I took him to various sites of malaria control operations throughout the State, many of which he had been instrumental in promoting and developing. He nearly walked my legs off in his almost insatiable desire to see more and more of our antimalarial projects, the majority of which were carried on under the auspices of the Works Projects Administration. We stayed one night in Americus, in Sumter County, where we were obliged to sleep in one of the circular rooms—five beds in each!—of the single tower of the Windsor Hotel, allegedly designed after the famed Windsor Castle of England, but on a vastly simpler and more modest scale. This interested him greatly as he was by birth an Englishman, though he later became a naturalized citizen of this country. I recall that he was greatly disturbed about his wife's health and went to much trouble to get into telephonic

communication with her before we retired. He was a kindly man, energetic and professionally expert. But above all, he possessed the most compulsive drive to advance malaria control of any individual I have ever known. He organized numerous school and adult groups in many places to develop knowledge about malaria and to promote its prevention. He was never too busy to respond to invitations to discuss the subject before business men's service organizations. Those who knew him well will recall that space left over at the end of his telegraphic messages was always thriftily utilized to remind recipients of the perils of paludism and the costs of control.

And so I am deeply honored to receive this award—in the sober realization that whatever has been accomplished has been possible only with the help of my associates, all of us dedicated to the same antimalarial objective that Mr. LePrince pursued so enthusiastically and industriously. Again, my thanks to all concerned in its bestowal.

SIMIAN MALARIA IN MAN

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The efforts of the World Health Organization (WHO), the United Nations International Children's Emergency Fund (UNICEF), the International Cooperation Administration (ICA), the Pan American Health Organization (PAHO), and other international agencies have augmented the general acceptance of the technical feasibility of world-wide malaria eradication. The decision to launch this program was taken only after careful consideration of factors such as resistance to insecticides and plasmodicides, over-all and continuing costs, and the availability of qualified personnel, necessary equipment, and materials.

Curiously enough, in retrospect, it was taken for granted by malariological consultants that non-human reservoirs of malaria transmissible to man would not be a problem. This may still be true. Doubtless this inadvertence was due to (1) the lack of epidemiologic evidence of such a possibility, and (2) the difficulty experienced by the few investigators who have tried to transmit malaria parasites from subhuman sources to man. However, the recent occurrence of accidental human infections with *Plasmodium cynomolgi bastianellii*, as reported by Eyles, Coatney and Getz,[1] stimulated us to undertake epidemiologic studies related to clinical and parasitological aspects of this infection in man. The initial results of these studies are reported here.

MATERIALS AND METHODS

This investigation, carried out at the U.S. Penitentiary, Atlanta, Georgia, is divided into two parts. Twenty inmate volunteers, 7 Negroes and 13 whites, participated in part 1 and 5 volunteers participated in part 2. The men were between the ages of 21 and 35. They were screened for mental and physical fitness before admission to the project. This included a history and physical examination, chest x-ray, electrocardiogram, hemoglobin, hematocrit, white cell count, erythrocyte sedimentation rate, blood urea nitrogen, bromsulfalein, urinalysis, and a thick smear for malaria parasites. Inmates with a history of malaria or jaundice were excluded from participation. One white man who had been splenectomized some 5 years before was included because of special interest in the course of infection in such an individual.

Each of 20 volunteers was bitten by mosquitoes, never less than 25, heavily infected with *P. c. bastianellii* acquired from rhesus monkeys (*Macaca mulatta*). The biting episodes took place on 2, 15, and 23 June 1960 and involved 3, 2, and 15 volunteers, respectively. *Anopheles quadrimaculatus* was the vector for the first two episodes and *Anopheles freeborni* for the last one. The volunteers were exposed to infection by mosquito bite in groups of 3 men each, except the 15 June episode which involved 2 men only. Each mosquito was allowed to bite each man in a group according to a technique of interrupted feeding.[2] In an attempt to equalize the sporozoite dosage, the first mosquito was allowed to bite man A, then B, and finally C; the second mosquito bit man B, then C, and finally A, and so on.

Laboratory procedures, including those of the basic work-up, were repeated if indicated.

Beginning the 5th day after the biting episode, thick and thin blood smears were made daily from each volunteer and stained with Giemsa. Daily smears were continued through 75 days of observation and then twice weekly during a total observation period of 150 days, unless otherwise noted. Parasite estimations were based on the examination of 150 thick smear fields using the oil immersion objective and a 5 × ocular. This is approximately equal to the examination of 0.3 cmm of blood. Although this criterion was used in determining the prepatent period, many slides were examined more extensively.

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Volunteers were admitted to the hospital when smears first became parasite positive or when symptoms referable to malaria were evidenced. After admittance, oral temperatures were taken at 4-hour intervals when below 100°F and at hourly intervals when above 100°F.

Patients received analgesics, antihistamines, and mild sedatives when indicated. In severe cases of nausea and vomiting, phenothiazine derivatives were given. Every patient was examined daily, generally by the same observer, and the physical findings and symptoms evaluated and recorded. During the period of observation, symptoms referable to disease other than malaria were minimal.

In general, an infection was allowed to run its course in a given volunteer unless there were medical or administrative reasons for specific treatment. When such treatment was given, it was limited to a single dose of quinine sulfate (300 or 600 mg) or chloroquine (600 mg of the base) except in one case in which 1,500 mg (base) was given over a period of 3 days.

Man to man transfer of *P. c. bastianellii* via mosquito bite involved two volunteers. *A. freeborni*, infected as a result of feeding on one of the initial volunteers, were allowed to bite one of the above volunteers and an uninfected monkey on 2 August 1960. A similar biting episode which took place 6 days later involved a second volunteer and another monkey. Each volunteer was bitten by four infected mosquitoes as determined by postprandial dissection. Observations concerning these volunteers were as described earlier.

On the 8th day, blood was drawn from each of the volunteers and injected intravenously into two uninfected rhesus monkeys. This was repeated several times when the patient's symptoms suggested parasite activity. During one of these periods, blood was given to an additional volunteer. Finally, when parasites were demonstrated in the blood of the volunteer bitten on 2 August 1960, blood was transferred to an additional volunteer and to an uninfected monkey. A second volunteer received blood 4 days later.

The methods and observations employed in this study were similar to those employed in the study of human vivax malaria by one of us (G. R. C.) under identical conditions in respect to personnel, volunteers, and facilities. This permitted comparison of this infection, sometimes referred to as simian vivax, with *Plasmodium vivax*.

The fact that the accidental infections in man, as well as experimental infections in monkeys, responded quickly to specific antimalarial drugs gave assurance that the experimentally induced infections could be controlled.

RESULTS

Monkey-mosquito-man. Parasites were demonstrated in 12 of the 20 volunteers. The prepatent period ranged from 15 to 30 days with seven occurring at 19 days or earlier. The parasite density ranged from 5 to 500 per cmm. The low parasitemia and its evanescent expression did not permit a correlation between parasites and clinical manifestations except in two instances. Parasites were not demonstrated in the blood from any of the Negroes although three exhibited signs and symptoms consistent with proved infection in whites.

The various fever patterns for the first 75 of the 150 days of observation are shown in Figure 1. It is to be noted that: (1) the incubation period ranged from 15 to 23 days; (2) there was considerable variation in these patterns with the duration of an attack ranging from 1 day (in AD.) to 45 days (in RO.); (3) fever in five patients exhibited a fairly rhythmic tertian pattern beginning between the 19th and 23rd day; and (4) temperature elevations during the initial paroxysms were higher than subsequently. No paroxysms were observed beyond the 70th day except in one patient (RO.) who exhibited temperature elevations above 101°F on days 100, 104, and 113. All volunteers with patent parasitemia exhibited fever at sometime during the period of observation. Although none of the Negroes exhibited parasitemia, two had isolated fever elevations associated with clinical manifestations referable to malaria. The highest temperature observed was 105.6°F in patient KE. Chills occurred in 9 subjects; all were mild and of short duration.

The most frequent symptom was headache followed in decreasing frequency by anorexia, abdominal pain, joint pains, nausea, myalgia, back pains, vomiting, chest pains, chills, and cramping. Headache was generally described as an aching bilateral frontal pain, often extending to the back of the head and lateral

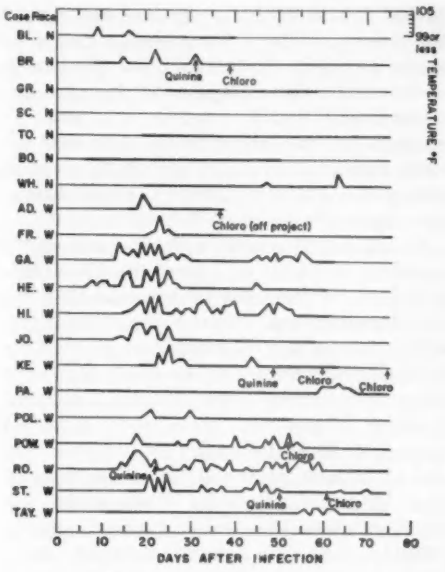

FIG. 1. Fever patterns observed in volunteers bitten by mosquitoes heavily infected with *Plasmodium cynomolgi bastianellii*.

aspects of the neck suggestive of a "tension headache." Abdominal pain was often in the right and/or left upper quadrants, and occasionally was increased with deep inspiration. On palpation, 14 patients exhibited tenderness in the liver area, while 13 showed tenderness in the splenic region. Anorexia, the second most frequent symptom, ranged from simple loss of appetite to a feeling of hunger until the first few bites of food were taken following which additional food could not be tolerated. Chest pain was present at some time in 11 subjects; occasionally this was also pleuretic in character and accentuated on inspiration. All symptoms were transitory, the subjects feeling much better on some days than on others.

The most significant physical findings were splenomegaly in 15 patients (12 whites and 3 Negroes), and hepatomegaly in 16 patients (13 whites and 3 Negroes). These enlargements were usually accompanied by tenderness. These signs appeared early in the infection and were inconstant from day to day.

The blood pressure readings were usually 110 to 120 systolic and 60 to 70 diastolic with individual variation. The pulse rate correlated with temperature.

Sixteen of the volunteers exhibited clinical responses ranging from moderate to severe (temperature above 100°F, enlarged tender liver or spleen and symptoms of moderate to severe intensity as graded 3 to 4 plus); two evidenced mild responses (temperature under 100°F, headache, nausea, and/or vomiting and anorexia); and two were asymptomatic.

Quinine was given to 4 people, as shown in Figure 1. The earliest was day 22 (in RO.). This patient had experienced 6 days of remittent fever as high as 104.8°F and relief was indicated. Following this medication he became afebrile and remained relatively so until day 31. Between day 30 and day 51, quinine was given to 3 other patients, to one for transitory relief of symptoms and to the other two for termination of the infection.

The splenectomized patient (POW.) exhibited a prepatent period of 30 days in contrast to the range of 15 to 21 days for the rest. In all other respects, the course of his infection offered no striking variation from the general pattern.

Man-mosquito-man. The progress of the infection in the first volunteer may, for ease of description, be divided into five periods: (a) 0 through day 23, (b) 24 through 31, (c) 32 through 70, (d) 71 through 109, and (e) 110 through day 150.

(a) The first complaint was headache on the 11th day of observation. On the 14th, the patient experienced generalized pains, especially in the upper abdomen, accompanied by a slightly enlarged tender liver and spleen. These mild signs and symptoms, not accompanied by fever, continued through the 23rd day. Smears were negative. Blood drawn on day 8, when the first circulating parasites could be expected, and injected into an uninfected monkey failed to produce an infection.

(b) The patient had fever of 101°F on day 24 and similar rises above 100°F occurred on two subsequent occasions. Moderate transitory headache, mild nausea and joint pains were the chief complaints. Smears were negative.

(c) The patient was essentially afebrile and asymptomatic with a barely palpable spleen. Smears were negative. Blood inoculated into monkeys failed to produce infections.

(d) There were 20 episodes of fever above 100°F of which 11 were 101°F or above. Of this

latter group, 5 occurred between day 105 and 109 with peaks above 102°F. These recurrent temperature elevations were more or less tertian in character and intermittent except for the last 4 which were remittent. The symptoms became more severe; vomiting occurred frequently, and the liver and spleen increased in size. Complaints of upper abdominal pain were more common. On day 77, when the temperature was 101°F, blood was transferred to another volunteer who, at this writing, has shown no evidence of infection. Parasites were demonstrated for the first time on smear on day 107. Blood transferred to a volunteer and to an uninfected rhesus monkey produced a patent infection in each.

(c) Following day 109, there were no febrile attacks and the patient was relatively symptom free. The spleen and liver became barely palpable; parasitemia with gametocytes was demonstrable during the early part of this period. Blood transferred to a volunteer on day 111 resulted in a patent infection.

Observations on the second volunteer produced information which can be divided into two periods: (a) 0 through day 27, and (b) 28 through day 150.

(a) The first complaints, beginning day 5, were headache of moderate severity, abdominal pain, anorexia, and vomiting. These symptoms were transitory through day 27. On day 9 there was temperature of 100.4°F with a barely palpable spleen. During the period, there were 13 episodes of fever above 100°F but less than 101°F. The spleen became tender on day 10 and continued in this condition through day 25. Smears were negative. Blood drawn on day 8 and injected into an uninfected monkey failed to produce an infection.

(b) During this period the patient was virtually asymptomatic and afebrile. There were only 4 episodes when the temperature rose to 100°F. Parasites were never demonstrated and blood transferred to uninfected monkeys failed to produce an infection.

DISCUSSION

According to Garnham,[3] *P. c. bastianellii* belongs to the *cynomolgi-vivax* group, the only difference between *P. c. cynomolgi* and *P. c. bastianellii* being in the "nature of the pre-erythrocytic schizogony." Among the 20 patients exposed to *P. c. bastianellii* by mosquito bite, 12 had typical vivax-like parasites but each of the 20 occasionally exhibited forms which could not be accepted as parasites. These questionable bodies might be analogous to the abnormal forms found by Garnham in the blood of monkeys. If later investigations show that the forms seen by us are in fact bastianellii parasites, then each of the 20 volunteers was patent sometime during the period of observation.

Of the eight patients without demonstrable parasites, two were asymptomatic. These subjects, both Negroes, were the only ones bitten on 15 June 1960, and although postprandial dissection showed that the mosquitoes were heavily infected, there is no way to prove the sporozoites were infective. The situation, however, is quite different with the six who exhibited symptoms. Five of the six were Negroes and each of them, as mentioned earlier, was included in a biting group made up of two whites and one Negro. Each of the white subjects, with one exception, became patent, providing fairly conclusive evidence that each of the Negroes in that group was actually infected. In retrospect, we probably should have anticipated such a result and arranged periodic transfer of the blood of each participant to uninfected monkeys. A case in point is that of subject H. A., bitten by more than 30 infected mosquitoes at our Memphis laboratory on 5 May 1960.[1] He experienced general malaise on 15 May with temperature elevations near 100°F on the 16th and 17th. Malaise continued through the next 3 days. When parasites could not be demonstrated, blood was withdrawn and inoculated into an uninfected monkey. This animal became parasite positive 6 days later. H. A. felt well until 21 July when he complained of headache. Slides were examined and no parasites were found. On 10 August, the 96th postinfection day, he experienced severe headache and nausea and a temperature of 99.4°F. Parasites could not be demonstrated but blood inoculated into an uninfected monkey produced an infection 19 days later.

The fact that none of the 7 Negroes exhibited a parasitemia whereas 11 of the 12 whites (91%) did so, corresponds to the generally accepted concept that Negroes are more refractory than whites to 'vivax' parasites. Young *et al.*,[4] working with several strains of *Plasmodium vivax*, found that the infection rate following mosquito bite

in Negroes was 27% as against 98% in whites. When one considers that this *cynomolgi-vivax* is normally a parasite of monkeys, the infection rate in our white subjects is remarkable.

The characteristics of the *P. c. bastianellii* infection observed among the 20 volunteers were variable, but they differ in several respects from those in human vivax. One significant difference is the relationship between blood parasitemia and fever during the primary attack. The parasite density in human vivax becomes detectable near the onset of fever and then increases rapidly to a peak of from 8,000 to 20,000 per cmm, occasionally up to 50,000 or greater. During the next 60 days, it gradually decreases to submicroscopic levels. During this over-all period, there may be 20 to 30 temperature elevations which are usually tertian in character. In patients infected with *P. c. bastianellii*, however, the highest parasite density was only 500 per cmm and this occurred in only two patients. The highest parasite counts occurred between the 9th and 14th day. Thereafter, the parasites were present only in extremely low densities. Temperature elevations with a tertian cycle occurred but with far less frequency than in vivax. The height of the fever during paroxysms was similar to what is generally seen in vivax but was not associated with violent shaking chills.

The mean prepatent period of 20.4 days and the mean incubation period of 17.4 days were longer than the corresponding periods for vivax, namely, 12.2 days and 13.4 days, as reported by Kitchen[5] in a summary of 428 naturally induced infections.

Nausea and vomiting, though present in both entities, seemed to be less pronounced and less severe in bastianellii than in vivax malaria.

Characteristically, headache associated with vivax may be described as a pounding, throbbing pain, located posterior to the eyes or deep within the cranial vault. The difficulties in objectively evaluating and describing subjective symptoms in a group of inmate volunteers in an open ward should be understood.

Abdominal pain, especially in the left upper quadrant, as well as hepatosplenomegaly appears earlier in bastianellii and is transient, rather than persistent as in vivax infections.

In general, the disease appears more benign in bastianellii malaria than in vivax, although its severity appears out of proportion to the degree of parasitemia.

As an extension of these studies, additional infections with *P. c. bastianellii* were induced by mosquito bite beginning in mid-October. Observations on these subjects include extensive laboratory studies along with continued clinical observations. Results to date confirm and expand present observations.

A question of special interest in malaria eradication is whether this infection is a zoonosis, defined by the joint WHO/PAHO Expert Committee on Zoonosis (1959) as: "Those diseases and infections which are naturally transmitted between vertebrate animals and man." The results of our studies show that *P. c. bastianellii* can be transmitted to man and from man to man by the bites of infected mosquitoes. In our experience, the first was accomplished with ease. The latter, although successful, was accomplished in only one out of two trials. In both trials, the mosquitoes harbored light infections and each subject was bitten by only 4 infected mosquitoes.

These results show that *P. c. bastianellii* qualified experimentally as a zoonosis, but whether it will be significant in terms of malaria eradication can only be determined by further investigations now in progress.

SUMMARY

Anopheles quadrimaculatus and *A. freeborni* mosquitoes which had acquired their infection from monkeys infected with *Plasmodium cynomolgi bastianellii* were allowed to bite 7 Negroes and 13 white inmate volunteers. No volunteer received less than 25 infected bites. Observations were as follows: (1) parasitemia of low density was demonstrable in 12 of the 13 white subjects; (2) the prepatent period ranged from 15 to 30 days with an incubation period of 15 to 23 days; and (3) parasitemia was not demonstrated in any of the Negroes, although 3 exhibited signs and symptoms referable to malaria.

Of the 20 volunteers, 16 exhibited clinical responses with oral temperatures over 100°F, enlarged tender liver or spleen and other symptoms graded from moderate to severe. Two exhibited temperatures under 100°F with mild symptoms, and two were asymptomatic. The most frequent symptom was headache followed in decreasing frequency by anorexia, abdominal

pain, joint pain, nausea, myalgia, vomiting, chest pain, chills, and cramping. The most significant physical findings were splenomegaly and hepatomegaly which appeared early in the infection and were transitory. In general, this disease was more benign than that generally encountered in vivax although the severity of the symptoms appeared out of proportion to the parasitemia.

Man to man transfer of infection with *P. c. bastianellii* was accomplished by allowing *A. freeborni* infected on man to feed on two human subjects. Each subject developed symptoms referable to malaria and one developed patent parasitemia on day 107. On that day blood transferred to another volunteer and to an uninfected rhesus monkey resulted in an infection in each. Blood transferred to man and to monkeys prior to day 107 gave negative results.

The results reported in this paper show that *P. c. bastianellii* qualifies experimentally as a zoonosis. In addition, we have demonstrated that man-mosquito-man transfer can also occur. Whether these findings will be significant in terms of malaria eradication will only be determined by further investigations.

ACKNOWLEDGMENTS

We wish to express our thanks to Mr. James V. Bennett, Director of the Federal Bureau of Prisons, and to Dr. H. M. Janney, the Medical Director, for permission to carry out these studies. We are also indebted to Mr. David M. Heritage, Warden of the U. S. Penitentiary, Atlanta, Georgia; Dr. Carl I. Pirkle, Chief Medical Officer; and their respective staff, whose interest and indispensable co-operation contributed to the success of these investigations.

We find it difficult to make adequate acknowledgment to the inmates who volunteered for this project. These men willingly bore the discomforts and uncertainties of this malaria, and submitted to the necessary laboratory procedures. We wish to extend our thanks to them.

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CHLOROQUINE RESISTANCE IN *PLASMODIUM FALCIPARUM*

MARTIN D. YOUNG* AND DONALD V. MOORE†

A strain of *Plasmodium falciparum* malaria apparently originating in Colombia, South America, shows a poor response to normal and above-normal doses of chloroquine.[1] The history of the patient (FEC) from whom the malaria was obtained indicates that he had his first attack of malaria in the Magdalena Valley, Colombia, in May, 1960, at which time he was treated with chloroquine, quantity unknown. On July 6, 1960, while in the United States he had malaria again and received 2.1 grams of chloroquine diphosphate. Five days after the beginning of that treatment, he still had asexual parasites in peripheral blood, at which time he began primaquine therapy, 15 mg daily for 14 days. No parasites were found on July 15th. On July 31st he had a return of symptoms. His blood was positive on August 5th. On August 7th infected blood from the patient was sent to Columbia, South Carolina, and injected into a neurosyphilitic patient. So far this malaria strain has been induced into eight patients, seven by infected blood and one by infected mosquitoes. Seven of these have been treated with chloroquine and the results are summarized in Table 1; some of the results are presented graphically in Figure 1.

Chloroquine diphosphate was used in the oral doses. Single doses of 150 mg and of 600 mg were given, as well as the standard 1.5-gram regimen over a 3-day period, namely, 600-mg and 300-mg doses on the 1st day, then single 300-mg doses on the 2nd and 3rd days. Chloroquine dihydrochloride was used for injection. The amount of chloroquine is expressed in terms of the base.

As shown in Table 1 and Figure 1, with the exception of one case the parasites either were not removed or, if removed, the infection relapsed shortly thereafter.

The giving of 400 mg chloroquine hydrochloride intramuscularly (no. 1334) only 3 days after 150 mg chloroquine diphosphate orally did not reduce the parasitemia, whereas in other strains 400 mg intramuscularly eliminated the parasitemia in about 3 days. The parasitemia was eliminated after 9.75 grams quinine given in 5 days but upon relapse 600 mg chloroquine did not remove the parasites. Two other patients (1307 and 1331) received 600 mg chloroquine in a single dose; 48 hours later the parasites had increased. In normal strains the parasitemias are eliminated in 3 days or less by this amount of drug (Table 1).

Four patients (1322, 1327, 1328, and 1335) were given 1.5 grams chloroquine. One patient (1327), a boy weighing 78 pounds, gave a normal response, the parasitemia being eliminated within 3 days. No relapse occurred within the following 29 days. In one patient (1322) the parasitemia was cleared in 3 days, but a relapse occurred 18 days after the beginning of treatment. In the other two cases the parasitemia was not eliminated. With the normal strains, parasitemias are eliminated within 3 days by 1.5 grams and the relapse rate is less than 2% (1 out of 57) (Table 1).

Two patients (1307 and 1331) both received the 1.5-gram regimen shortly after a single dose of 600 mg so that a total of 2.1 grams was given within 5 and 7 days, respectively. The parasites were removed slowly, namely, 8 and 10 days after the first 600-mg dose, respectively; and relapses occurred after 9 and 16 days, respectively. The response to the total 2.1 grams was slower than that seen in normal strains to lesser dosages of 600 mg and 400 mg (Table 1).

One patient (1335) was infected by mosquitoes which had acquired the infection from patient 1334. When the infection in patient 1335 was challenged by 1.5 grams chloroquine given in 3 days, the parasites were not cleared from the blood stream and he was still positive 27 days later. This indicates that the resistance is transmitted through the sporogonous cycle in the mosquito.

The parasitemias of susceptible strains are removed in less than 3 days by 1.5 grams chloro-

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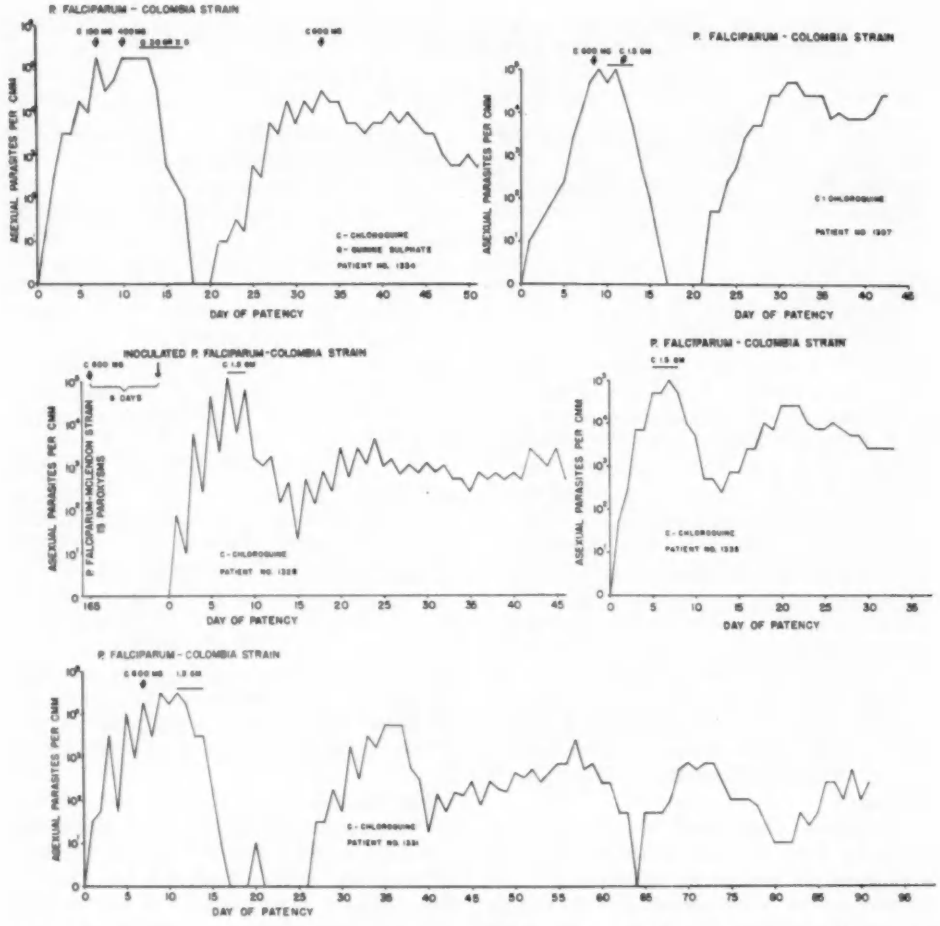

FIG. 1. The response of *Plasmodium falciparum* (Colombia strain) to different dosages of chloroquine

quine and the relapse rate is less than 2% (Table 1). Most of the Colombia strain infections treated with this dosage were either removed more slowly than the susceptible strains, or not cleared at all. Furthermore, of the four cleared by 1.5 grams or 2.1 grams, three relapsed in 9 to 18 days.

A compilation of 622 cases from North America and Malaya shows that in normal (susceptible) strains the parasitemias are eliminated in 3 days or less by either 0.4 gram or 0.6 gram chloroquine given in a single dose orally (Table 1). None of the three patients with the Colombia strain were cleared by 0.6 gram.

Plasma was obtained from most of the patients and the chloroquine level determined by the method of Brodie *et al.*[5] The results shown in Table 1 indicate that for those receiving 600 mg or more of the drug, the plasma level was above the range of that usually considered sufficient to eliminate the parasites, namely, 10 μg per liter.[6] It is interesting that the highest concentration was found in the patient (1327) weighing only 78 pounds, who gave a normal response and who did not relapse during the following 29 days. The level was at the upper limits of those usually obtained after therapeutic amounts of the drug.

Urine was obtained from most of the patients

TABLE 1

Response of Plasmodium falciparum *(Colombia strain) to chloroquine (oral doses except as otherwise noted)*

Patient no.	Chloroquine regimen (base)	Chloroquine in plasma		Days to neg. blood	Days to relapse	Remarks
		Days after 1st dose of latest regimen	μg/L*			
1334	150 mg s.d.†			Parasites increased		1st ℞, parasite density 105,000/cmm
1334	400 mg i.m.‡	1	42	Parasites increased		2nd ℞, parasite density 139,000/cmm 3 days after 1st ℞
1334	Q§ 650 mg tid × 5			6	10	3rd ℞, parasite density 224,000/cmm 2 days after 2nd ℞
1334	600 mg s.d.	1	131	—		4th ℞, still positive 35 days later‖
		7	130			
1307	600 mg s.d.	1	130	Parasites increased		2 days later 2nd ℞ started
1307	1.5 g¶ in 3d**	2	326	6	9	Still positive on relapse day 24‖
		9	389			
1331	600 mg s.d.	2	103	Parasites increased		4 days later 2nd ℞ started
1331	1.5 g in 3d	2	530	7	16	Still positive on relapse day 64††
		12	192			
1322	1.5 g in 3d			3	18	Still positive on relapse day 56††
1327	1.5 g in 3d	2	585	3		Still negative 29 days after ℞, wt. = 78 lb
		7	570			
1328	1.5 g in 3d	2	209	—		Previously had 15 fevers McLendon *P. falciparum*, still positive 47 days after ℞‖ ††
		9	118			
1335	1.5 g in 3d			—		Infected by mosquitoes from 1334, still positive 27 days later††

Comparative data (average) normal strains

Strain	Chloroquine regimen	Days to neg. blood	Days to relapse	Remarks	References
Pan‡‡ & S.C.§§	1.5 g in 3d	2.8	47	1 of 57 relapsed	2, modified
Pan & S.C.	0.4 g i.m.	3.2	34	4 of 20 relapsed	2, plus additional data by Young
Pan & S.C.	0.6 g s.d.	3.0		0 of 2 relapsed	Previously unreported (Young)
Panama	0.6 g s.d.	1.2	NI‖‖	388 cases (6 failures)	3
Malaya	0.6 g s.d.	2.5	NI	114 cases	4
Panama	0.45 g s.d.	1.6	NI	80 cases (5 failures)	3
Malaya	0.4 g s.d.	2.3	NI	40 cases	4

* μg/L = micrograms per liter.
† s.d. = single dose.
‡ i.m. = intramuscular.
§ Q = quinine.
‖ Treated with another drug.
¶ g = gram.
** d = days.
†† As of December 13, 1960.
‡‡ Pan = Panama.
§§ S.C. = South Carolina, U.S.A.
‖‖ NI = no information.

1 and 7 days after drug administration and all gave a positive Haskins test for chloroquine.[7]

One patient (1328) had just finished a primary attack of 15 paroxysms with a South Carolina (McLendon) strain of *P. falciparum*. He was given 600 mg single dose of chloroquine. Eight days later he was inoculated with blood infected with the Colombia strain of *P. falciparum* from patient 1307; this was 2 days after the latter had received 600 mg chloroquine. The Colombia infection became patent 2 days after inoculation. The acute course of this infection indicated that little, if any, cross-immunity existed from the preceding South Carolina strain infection.

DISCUSSION

Six out of seven cases of *P. falciparum* either failed to respond adequately to or relapsed quickly after normal doses of chloroquine. Two patients who had received a total of 2.1 grams did not respond as well as do patients infected with normal strains when given 600 or 400 mg, that is, one-third or one-fifth as much. This could be interpreted to be at least a five-fold resistance.

The fact that the drug was given under close supervision, that the urines gave a positive Haskins test for chloroquine, and that the plasma levels of the chloroquine were at therapeutic concentrations eliminates any doubts that the drug was being delivered to the parasites in the blood stream.

The consistency and quality of the poor response of this strain to chloroquine indicate that the strain is resistant to that drug.

So far as we know, this is the first time that resistance of human malaria to chloroquine has been shown definitely. Chloroquine is widely used throughout the world in the treatment and suppression of clinical malaria. It is being used in malaria eradication programs as a complementary measure and in special situations, where insecticiding measures are not satisfactory or feasible, as the main attack on malaria. The occurrence of resistance to this drug by the malaria parasite appears to have important implications in the chemotherapy of this disease.

The extent of the drug resistance by *P. falciparum* and the conditions under which it appears should be investigated. Whether other species of human malaria have become resistant should be determined.

SUMMARY

The response to chloroquine of a strain of *Plasmodium falciparum* malaria from Colombia, South America, has been studied in seven patients. Six of these infections responded poorly to normal (0.6 gram, 1.5 grams) and above normal (2.1 grams) doses of the drug. The parasites were either not cleared from the blood stream or were eliminated slowly, after which they relapsed quickly. In two instances, 1.5 grams of chloroquine failed to eliminate the parasitemia; and, in two other instances, the parasites increased after a 600-mg single dose.

These findings indicate that this strain of malaria is resistant to the usual doses of chloroquine. The resistant quality was still present after mosquito passage.

Because of the widespread use of this drug for the suppression and cure of malaria and especially in malaria eradication programs, the occurrence of resistance by the parasites appears to be of considerable importance.

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A POSSIBLE MECHANISM OF PROLONGED ANTIMALARIAL ACTIVITY

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In man chloroquine is slowly metabolized and excreted, and a single dose of 600 mg (as base) of this antimalarial drug can provide suppression for at least 20 days.[1] Individuals receiving single weekly doses of 300 mg base for 26 weeks experienced suppression for at least 16 days after the last treatment.[2] Yet in a program where drugs are used for the eradication of malaria, even longer parasite-free intervals would be desirable. Since chloroquine exerts its antimalarial activity directly in vivo, a prolongation of this activity might be obtained if its metabolic transformation could be retarded.

Titus *et al.*[3] isolated several metabolites from the urine of human volunteers given chloroquine; desethylchloroquine and a deaminated product were present in large concentrations. McChesney *et al.*[4] confirmed the work of Titus regarding urinary metabolites and established that desethylchloroquine, a compound arising from N-dealkylation, was the principal metabolic product. Apparently, the primary pathway for chloroquine metabolism in man is by N-dealkylation.

This metabolic transformation of chloroquine is certainly not unique, for many drugs which are alkylamines undergo dealkylation in the body. For example, aminopyrine,[5] ephedrine,[6] morphine,[7] meperidine,[8, 9] methylamphetamine[10] and mephobarbital[11, 12] are all demethylated in vivo.

The biochemical mechanism responsible for the removal of alkyl groups was studied in greater detail by various investigators. Liver enzyme systems for the dealkylation of aminopyrine and other alkylamines were demonstrated by LaDu *et al.*[13] In the rabbit the enzyme activity was localized in the microsomal fraction of the cell, and had the requirements of reduced triphosphopyridine nucleotide (TPN-H) and oxygen. These enzyme systems, as shown by Gaudette and Brodie,[14] are highly non-specific and will oxidatively dealkylate a whole spectrum of compounds. The metabolism of some of these compounds could also be inhibited by β-diethylaminoethyl diphenylpropylacetate·HCl (SKF 525-A).

This report describes the effect of SKF 525-A on plasma levels of chloroquine in rabbits. The results of metabolic studies in rabbit liver homogenates with SKF 525-A and various other agents are given, and the ability of some of these agents to prolong the plasma level of chloroquine is also described.

MATERIALS AND METHODS

Materials

Compounds used as inhibitors of chloroquine metabolism in vivo and in vitro included β-diethylaminoethyl diphenylpropylacetate·HCl (SKF 525-A), isopropyl-1-isonicotinyl hydrazine (iproniazid), isonicotinyl hydrazine (isoniazid), 2,4-dichloro-6-phenylphenoxyethyldiethylamine·HCl (Lilly 18947), pyrimethamine·HBr, suramin, triazine, amodiaquin·HCl, quinacrine·HCl, hydroxychloroquine sulfate, desethylchloroquine·HCl, primaquine diphosphate and pamaquine naphthoate.

Methods

Analytical. Chloroquine was determined by the fluorescence procedure of Brodie *et al.*[15] except that the compound in biological materials was initially extracted into heptane from pH 9.0, and the final extract was assayed in the Aminco-Bowman Spectrophotofluorometer in a solution of 1N NH_4OH. The wave lengths of peak activation and fluorescence were 330 mμ and 400 mμ, respectively.

Chloroquine recoveries of 95% were obtained under these conditions. Standards and blank controls were prepared and handled in the same manner as unknowns for each experiment. A relative fluorescence of 50% was obtained when 50 μg of chloroquine diphosphate was run through the method. A reagent blank never gave a relative fluorescence of more than 2%.

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Plasma levels. In studies of plasma level determinations of chloroquine, control animals receiving the same concentration of the inhibitor alone were also included. Plasma samples from these animals were prepared and handled in the same manner as those treated with chloroquine and chloroquine plus inhibitor.

Blood samples were taken by cardiac puncture. Potassium oxalate was used as anticoagulant at a concentration of 1 mg/ml of blood. Three to five ml of the separated plasma was extracted and assayed for chloroquine. Hemolyzed samples were discarded. In most cases a fluorescence no greater than the normal blank was obtained with the plasma from animals receiving the inhibitor alone. In those studies involving hydroxychloroquine, values were corrected for fluorescence arising from this compound.

Preparation of tissue samples. Male albino rabbits were killed by displacement of the cervical vertebrae, and livers were immediately removed and chilled. The tissue was homogenized with a Waring Blendor at 0 to 3°C in 2 volumes of 0.1 M phosphate buffer, pH 7.4. The supernatant fraction employed in most of the studies was prepared free of unbroken cells, nuclei and mitochondria by centrifuging the homogenate at 9,000 × G in an International Refrigerated Centrifuge for 20 minutes.

For incubation studies of drug metabolism, 3 ml of the homogenate preparation, 100 μmoles of nicotinamide, 75 μmoles of $MgCl_2$, 0.2 μmole of TPN, 30 μmoles of glucose-6-phosphate, 100 μg of chloroquine diphosphate and 0.2 M phosphate buffer, pH 7.4, to a final volume of 5.0 ml, were placed in a 30-ml beaker. The reaction was carried out in a Dubnoff-Aminco metabolic shaker at 37°C for 1 hour in an atmosphere of oxygen.

Enzyme activity was assayed by measuring the disappearance of substrate.

RESULTS

Plasma levels of chloroquine in the rabbit. Initially, plasma levels of chloroquine were determined in the rabbit. A dose of 100 mg of chloroquine diphosphate was administered orally by stomach tube to each of three rabbits. Plasma levels were determined at .5, 1, 3, 4 and 6 hours. In three other rabbits, plasma levels were determined for the same time period after oral administration of 100 mg of both chloroquine diphosphate and SKF 525-A to each experimental animal.

As shown in Figure 1, animals given SKF 525-A with chloroquine diphosphate demonstrated a higher and more prolonged plasma level than those receiving chloroquine alone.

In another group of experiments, plasma levels were determined through the 6th day following a single dose of 100 mg of chloroquine alone in one group of three animals. In another group, plasma levels were determined after the animals had received a combination of 100 mg of the antimalarial with 100 mg of SKF 525-A. It is apparent from Figure 2 that the animals receiving the combination of drugs experienced levels of the antimalarials for a longer period of time. Cooper *et al.*[16] have demonstrated that SKF 525-A prolongs plasma levels of drugs that are metabolized by enzymes present in the liver.

Chloroquine metabolism by rabbit liver homogenates. Chloroquine metabolism was compared in the whole homogenate and in the 9,000 × G fraction after a 24-hour dialysis with and without the required co-factors for the TPNH production. The results are summarized in Table 1. The enzyme activity was localized in the 9,000 × G supernatant fraction and the co-factor requirements were the same as those necessary for the metabolism of many other alkylamine drugs.[13]

Effect of SKF 525-A on chloroquine metabolism. Since the metabolism of many of the alkylamine drugs is sensitive to inhibition by SKF 525-A, it was advisable to test inhibitory effects of this compound on the metabolism of chloroquine. The study was carried out with a fortified 9,000 × G fraction of rabbit liver homogenate. An inhibition of 57% was produced by SKF 525-A at a concen-

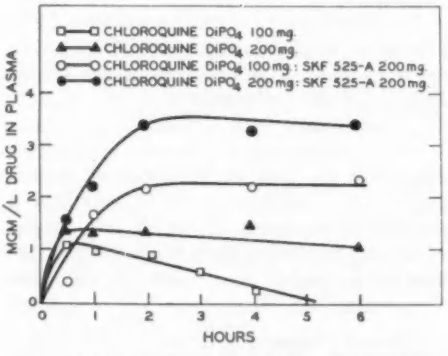

FIG. 1. The effects of SKF 525-A on the plasma levels of chloroquine in the rabbit.

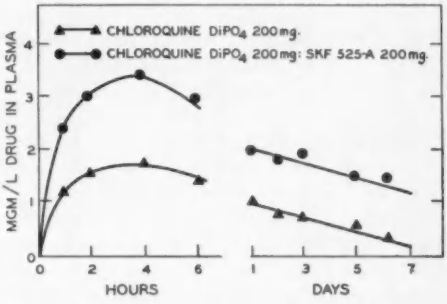

FIG. 2. SKF 525-A and the extent of prolongation of chloroquine plasma levels in the rabbit.

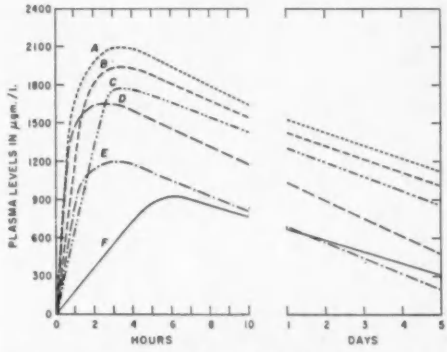

FIG. 3. Plasma levels of chloroquine in the rabbit receiving 100 mg of each drug in the combinations indicated: A, chloroquine—amodiaquin; B, chloroquine—hydroxychloroquine, chloroquine—pamaquine; C, chloroquine—primaquine; D, chloroquine—SKF 525-A; E, chloroquine, chloroquine—triazine, chloroquine—isoniazid, chloroquine—suramin, chloroquine—Lilly 18947, chloroquine—pyrimethamine; F, chloroquine—quinacrine.

tration of 1×10^{-5} M. Complete results are given in Table 2.

Other inhibitors of chloroquine metabolism in vitro. With the information that chloroquine metabolism could be inhibited by SKF 525-A, it seemed worthwhile to apply this same principle to compounds known to have a more extended half-life in the intact animal. The results of trials in this direction are given in Table 3. A perusal of the data shows that pyrimethamine, amodiaquin, quinacrine and primaquine are as effective as SKF 525-A as inhibitors of chloroquine metabolism.

Iproniazid and Lilly 18947 (2,4-dichloro-6-phenylphenoxyethyldiethylamine·HCl) inhibited chloroquine metabolism at a higher concentration, 1×10^{-4} M. Isoniazid was inactive at a concentration of 1×10^{-3} M.

General inhibitors to chloroquine metabolism in vivo. SKF 525-A would not have practical application as a prolonging agent. Axelrod *et al.*[17] have shown that the compound is eliminated from the body within 48 hours. An examination of the plasma level curves in Figure 2 indicates that the inhibitory effects of this compound were present only during the first 24 hours after treatment. A higher concentration is present in the animals receiving the combination since, initially, a higher level was obtained. However, the rate of metabolism of chloroquine in the two groups of animals, thereafter, was the same, as evidenced by the slope of the plasma level curves.

In the in vitro system a number of other compounds were found to inhibit the metabolic transformation of chloroquine as effectively as SKF 525-A. In screening a variety of these drugs for inhibitory activity in the intact animal, particular consideration was given to other antimalarials.

TABLE 1

Chloroquine metabolism by rabbit liver homogenate

Conditions	Chloroquine metabolized* (μg)
Whole homogenate	30
9,000 × G supernate	34
Dialyzed 9,000 × G supernate	8
Dialyzed 9,000 × G supernate + cofactors, TPN, glucose-6-phosphate, nicotinamide and magnesium sulfate	47

* 100 μg of chloroquine diphosphate incubated for 1 hour with approximately 1 g of tissue homogenate.

TABLE 2

Effects of SKF 525-A on chloroquine metabolism

SKF 525-A concentration	Chloroquine metabolized* (μg)	Inhibition (%)
0	44	
1×10^{-5} M	25	57
1×10^{-3} M	0	100

* 100 μg of chloroquine diphosphate incubated for 1 hour with fortified liver homogenate.

TABLE 3

General inhibitors to chloroquine metabolism

Inhibitor	Concentration	Chloroquine metabolized* (μg)	Inhibition (%)
—	—	52	—
SKF 525-A	1×10^{-5} M	26	50
Isoniazid	1×10^{-3} M	53	0
Iproniazid	1×10^{-4} M	36	31
Lilly 18947	1×10^{-4} M	37	29
Amodiaquin	1×10^{-5} M	21	60
Quinacrine	1×10^{-5} M	33	37
Primaquine	1×10^{-5} M	23	56
Pyrimethamine	1×10^{-5} M	32	38

* 100 μg chloroquine diphosphate was incubated for 1 hour with fortified liver homogenate in the presence of indicated concentrations of inhibitor.

These compounds, in many cases, would possess the additional quality of being very slowly metabolized and, consequently, able to exert inhibitory effects over a more prolonged period of time.

The antimalarial, chloroquine, and the prolonging agent were administered to rabbits orally at equal concentrations, and plasma levels were determined as previously described. The results of the study are summarized in Figure 3. The inhibitory effects in the intact animal did not always parallel the results obtained in the in vitro system. Compounds like Lilly 18947 and pyrimethamine, which were inhibitors in vitro, did not influence the chloroquine plasma level in vivo. However, various physico-chemical factors, as pointed out by Brodie and Hogben,[18] often alter drug action in vivo. Some of the antimalarials did prolong the levels of chloroquine. The most effective of these compounds were amodiaquin·HCl, hydroxychloroquine sulfate, primaquine diphosphate and pamaquine naphthoate.

DISCUSSION

In man the effectiveness of chloroquine as an antimalarial is limited only by its metabolic transformation, for little is excreted as unchanged drug. The metabolic transformation which chloroquine undergoes in the body was demonstrated by the identification of urinary metabolites. The amounts of desethylchloroquine found suggest that N-dealkylation is primarily the pathway of detoxication. Many other drugs which are alkylamines are detoxified in man and other mammals in an identical manner. Plasma levels of these drugs have been prolonged in the intact animal by administration of the drug in combination with SKF 525-A, an inhibitor to the metabolic transformation of these alkylamine drugs. The possibility that a drug combination might extend the therapeutic plasma levels of an antimalarial is particularly important to a program for the eradication of malaria.

The results in the present study show that enzyme systems of rabbit liver homogenates for the dealkylation of a spectrum of N-alkylamine drugs are also active on chloroquine. In addition, chloroquine metabolism is sensitive to inhibition by SKF 525-A. This compound is a potent inhibitor to chloroquine metabolism in vitro. An inhibition of 50% is achieved at an SKF 525-A concentration of 10^{-5} M.

This inhibition of chloroquine metabolism can also be achieved in vivo. As a result of the rate of biotransformation being slowed, the plasma levels of chloroquine can be extended in time and, presumably, a prolongation of malaria suppression would also be obtained. However, although SKF 525-A can effectively prolong the presence of chloroquine in the blood, such a drug combination would have little practical application.

An inspection of the plasma levels of animals receiving the drug combination and those receiving the antimalarial alone clearly indicates that the inhibitory effects of SKF 525-A are not present beyond the first 24 hours after administration of the drug. During this initial period, animals which have been treated with both drugs build up a blood concentration of the antimalarial. At the end of this time, the rates of elimination, as indicated by the slopes of the curves, become identical. Only the initial rate of metabolism is altered. These results would be anticipated from the findings of Axelrod *et al.*[17] that SKF 525-A is completely eliminated from the body within 48 hours. It would be more desirable to have a compound, not necessarily as strong an inhibitor, but more slowly metabolized so as to exert a more lasting effect.

Of the other compounds also inhibitory to the metabolism of chloroquine in vitro, several would have the same limitations as SKF 525-A. The drugs iproniazid and Lilly 18947 are also rapidly metabolized in the intact animal. However, some antimalarials are equally effective inhibitors to

the biotransformation of chloroquine. The compounds amodiaquin, primaquine, quinacrine and pyrimethamine inhibit chloroquine metabolism at concentrations which could be achieved in the body. If this inhibition can be achieved in vivo, these drugs would also possess the desirable characteristics of being slowly metabolized and excreted.

Apparently, other factors must also be considered. Relatively effective inhibition to chloroquine metabolism in the in vitro system is obtained with the compounds Lilly 18947 and pyrimethamine. However, the plasma levels of chloroquine are not extended with these drug combinations. It is true that the Lilly compound is readily metabolized in the intact animal, but pyrimethamine is only slowly metabolized and excreted. The physiological disposition in the intact animal of any potential inhibitor evidently also must be considered.

Other compounds investigated in this study appear to offer more promising combinations. The most effective were chloroquine in equiconcentration with either amodiaquin, hydroxychloroquine or pamaquine. With these combinations, elevated and prolonged plasma levels of the chloroquine are obtained.

These inhibitors of chloroquine metabolism would also have the advantage and desirable characteristics of being more slowly metabolized and excreted and, in addition, of possessing, in themselves, antimalarial properties. Studies assessing antimalarial activity in man and experimental animals are currently under investigation.

SUMMARY

An enzyme system in the supernatant fraction of rabbit liver homogenate for the metabolism of chloroquine is described. Various compounds, including SKF 525-A (β-diethylaminoethyl diphenylpropylacetate·HCl) and a number of antimalarial drugs, constitute effective inhibitors in vitro to the metabolic transformation of chloroquine.

Plasma levels of chloroquine can also be prolonged in experimental animals with some of these compounds. The most effective drugs tested include amodiaquin, pamaquine, hydroxychloroquine and primaquine.

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SEVEN YEARS' MALARIA RESEARCH AND RESIDUAL HOUSE SPRAYING IN NETHERLANDS NEW GUINEA

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New Guinea, the world's biggest island, is situated in the tropics north of Australia. The western Dutch part is sparsely populated by about 700,000 Papuans, a Melanesian race. Villages are small and, as a rule, inhabited by only 100 to 300 people. The economy is still largely a subsistence one, though impressive education and development projects are in operation or are in the planning stage. An estimated 350,000 inhabitants are living in malarious areas. Notwithstanding this small number of persons at risk, almost every aspect of malaria epidemiology can be found. In the southern part of the territory dry and wet seasons alternate and transmission, therefore, is seasonal. Over most of the territory, however, the rains may continue beyond the normal rainy season so that malaria transmission is perennial.

Malaria is holoendemic in some inland plains,[1, 2] hyperendemic in some coastal areas, mesoendemic on coral islands off the shore, and hypoendemic in mountainous areas. In the central mountains, anophelism without malaria occurs above 1,600 meters.[3] The area around the Wissel Lakes (1,700 meters) in the central mountains is free of anophelines.

The main vectors are *Anopheles punctulatus*, *A. farauti*, and *A. koliensis*, all three members of the *punctulatus* group of anophelines which is widespread throughout the territory.[4] They are highly antropophilic but relatively short lived.[2, 5] In general, they prefer to bite out-of-doors, but do not hesitate to come inside houses for a blood meal. Between blood meals they usually rest out-of-doors.[6] Of other proved vectors *A. karwari* is only of local and *A. bancrofti* only of minor importance.[2, 7]

After preliminary studies in experimental houses,[6] residual house spraying was started in 1954 in a pilot area. For this, the basin of Sentani Lake was chosen. The Nimboran Valley, southwest of Sentani, served for 1 year as a control area.[8] After 1 year of observations, residual spraying was gradually extended over larger parts of the territory, including the Nimboran Valley. Early in 1960, spraying had been completed in areas inhabited by 180,000 people. Results of malaria research and control work in Netherlands New Guinea since 1953 are presented in this paper.

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METHODS

With few exceptions, houses were sprayed twice a year with DDT at a rate of 2 g/sq m. Early during the campaign dieldrin was used in two areas. The residual action of this insecticide was, however, disappointing, as 80% of the anophelines escaped death 5 months after application.[9]

In 1958, residual spraying was combined with mass distribution of drugs. Van Dijk[10] had shown that in a sprayed district reduction of parasite rates could be accelerated considerably by eight distributions of chloroquine, at weekly intervals, to the entire population. In 1958, drugs were distributed three times to all residents in sprayed regions, and where necessary, two more times in 1959. In areas sprayed for the first time in 1959, drugs were distributed twice. To compensate, at least partly, for the small number of drug treatments, chloroquine was combined with the long-acting and sporonticidal pyrimethamine. Because of the low average weight of Papuans, dosages were fixed for adults at 450 mg chloroquine base and 50 mg pyrimethamine, and for children according to age. On an average, the attendance was 90% at each distribution.

The combination of spraying and drug distribution has been disappointing in some areas. In these areas, since July, 1959, the houses have been sprayed every 4 months with a mixture of 0.125 g dieldrin and 2 g DDT/sq m.

In all areas, evaluation surveys were made at a minimum interval of 3 months after the last drug distribution.

FIG. 1. The study areas in Netherlands New Guinea

By far most of the entomological and parasitological observations were done in the pilot and control areas, which, therefore, will be discussed separately. In the other regions, investigations were restricted to surveys. Geographical names can be found on the map in Figure 1.

OBSERVATIONS

Pilot and control areas. In Sentani, villages are situated either on the shore of a big lake or on small islands. The latter, of little interest, will not be discussed. Prior to spraying in the shore as as well as in the Nimboran villages the parasite rates were comparable to those described by Wilson[12] and by Wilson and Wilson[13] from a highly endemic area in Africa, which area, according to a classification proposed at the Kampala Conference,[11] is holoendemic. Under this Kampala classification, the spleen rate in holoendemic areas should be low in adults. However, in the Nimboran Valley and in Sentani this is not the case. A comparison of absolute parasite rates and spleen rates between the New Guinea and African populations is given in Table 1, which emphasizes the similarity in the former and the difference in the latter.

A more critical measure of the degree of endemicity than parasite and spleen rates is the number of malaria infections occurring in a population. It could be calculated that in the Nimboran Valley there were about 150 bites by sporozoite-positive anophelines per adult per year. For Sentani, this number was estimated to be about 70. Transmission rates such as these give rise to the highest possible degree of malaria endemicity.

The discrepancy between spleen rates in Africans and Papuans was discussed by Metselaar.[1, 2] It was concluded that the spleen rate is not a suitable yardstick for malaria endemicity. The assumption was made that the sickle-cell trait, frequently found both in Africans and in Indians living in highly endemic areas, is, at least partly, responsible for the low spleen rates of adults in these areas. The trait is not found in Papuans.[14] A new classification of

TABLE 1

Absolute parasite rates and spleen rates in Papuans from Sentani and Nimboran, and in Digo Bantu, before spraying

Absolute parasite rates (%)					Spleen rates (%)				
Age groups	Sentani 1953	Nimboran 1954*	Age groups	Digo Bantu†	Age groups	Sentani 1953	Nimboran 1954*	Age groups	Digo Bantu
0–2 mo	4	32			0–2 mo	27	73		
3–5 mo	53	73	Under 1 yr	87.5	3–5 mo	65	78	Under 1 yr	89
6–8 mo	59	77			6–8 mo	70	90		
9–11 mo	82	87			9–11 mo	64	100		
1 yr	84	88	1 yr	100	1 yr	88	96		
2 yr	80	91	2 yr	100	2 yr	90	91	1–5 yr	93
3–5 yr	73	90	3–5 yr	98.5	3–5 yr	97	99		
6–8 yr	66	82	6–8 yr	81	6–8 yr	99	98	6–10 yr	77
9–11 yr	46	72	9–12 yr	72	9–11 yr	99	99		
12–14 yr	35	62	13–16 yr	59	12–14 yr	99	97	11–20 yr	44
15–24 yr	29	41	17–25 yr	52	15–24 yr	88	95		
25–34 yr	26	36	Over 25 yr	44	25–34 yr	90	90		
35–44 yr	28	42			35–44 yr	84	85	Over 20 yr	39
Over 45 yr	32	28			Over 45 yr	82	78		

* Combined results of two surveys.

† After Wilson,[12] average of 10 bimonthly examinations.

malaria endemicity based on the absolute parasite rate was proposed by Metselaar and van Thiel[15, 16] at the Lisbon Conferences of Tropical Medicine and Malaria. That the peak in the parasite rate-age curve in Digo-Bantu was higher in Papuans was probably due to the famine conditions which prevailed in Tanganyika at the time Wilson performed his examinations. Because of the high number of infections, Sentani is regarded as a holoendemic area also, notwithstanding the fact that the peak reaches only 84%. Because of fish from the lake, nutrition is reasonably satisfactory in Sentani and resistance to infections, thereby, may be greater.

Early in 1954, villages in Sentani and surrounding areas were sprayed for the first time. A resurvey was made each year. In 1958, the population received four drug treatments (one extra). During the 1959 resurvey, which covered practically the entire population (about 2,500 individuals in the holoendemic area, a fifth drug distribution took place, while the positives, in addition, received chloroquine for 3 successive days, completing a full course (1,350 mg base for adults). Later in 1959, tablets were distributed a sixth time. Two villages with high absolute parasite rates were sprayed every 4 months with a combination of insecticides, and the inhabitants received drugs a seventh time. As a result of all these efforts, the absolute parasite rate dropped considerably; in 1960 it was only 1.5%.

The Nimboran villages were sprayed for the first time in 1955 with dieldrin. In 1957 the insecticide was changed to DDT. People received drugs five times. Since July, 1959, the area has been sprayed every 4 months with the DDT-dieldrin mixture. Although there was an impressive drop in parasite rates, no improvement was seen in 1960 over 1959. In Table 2 the results of several surveys in Sentani and the Nimboran Valley are summarized. Because of the low number of positives in Sentani in 1960, some age groups are combined. The decrease in the absolute parasite rates in Sentani is illustrated in Figure 2.

A curious change occurred in the ratio of the three malaria parasites in the Sentani area during the course of the spraying campaign. If transmission is almost stopped by residual spraying, one may expect that *Plasmodium falciparum* will be the first to disappear in the human reser-

TABLE 2

Absolute parasite rates in the Sentani pilot area and in the Nimboran Valley before spraying and several years after spraying started

Age groups (in years)	Sentani					Nimboran			
	Before spraying	1956	1958*	1959	1960	Before spraying	1958*	1959	1960
Under 1	43	3	3	4	0.9	69	6		10
1	84	12	10	8		88	25		6
2	80	25	13	6	3.1	91	28		5
3–5	73	37	13	9		90	38		22
6–8	66	46	20	7		82	47		15
9–11	46	30	21	8	1.6	72	40		8
12–14	35	22	19	9		62	33		11
15–24	29	14	13	4	0.9	41	22		16
25–34	26	7	7	4	1.1	36	16		10
35–44	28	12	5	5		42	11		10
Over 44	32	9	6	2	0	28	17		1
Average.....	46	20	12	5.7	1.5	61	28	10.5†	10.6

* Before drug distribution.

† The sample was not divided into the usual age groups.

voir and *P. malariae* the last. As soon as the reservoir is at its lowest and some residual transmission remains, *P. vivax* should have the best chance to regain its former relative position because of its short extrinsic cycle. However, as shown in Table 3, only 2 years after spraying started, *P. falciparum* came to the fore again. The phenomenon is discussed more extensively elsewhere.[17] It was found that in *P. falciparum* infections, probably because of a more rapid loss of immunity, gametocyte rates increased sooner than in infections with the other parasite species. The chances for anophelines to become infected by *P. falciparum* thus are greater and compensate for the shorter extrinsic cycle of *P. vivax*. Only during the last year, when no transmission occurred at all, did *P. vivax*, because of the frequency of its relapses,[18] become predominant. In 1960, gametocytes were not found in the few and presumably old *P. falciparum* infections. *P. malariae* was completely eliminated from the blood by the drug distributions, which was the case also in the Nimboran Valley. It is possible that some relapses may occur later.

Table 4 and Figure 3 show that the spleen rate did not decline as rapidly as did the parasite rates.

The saddle shape of the lower curves of Figure 3

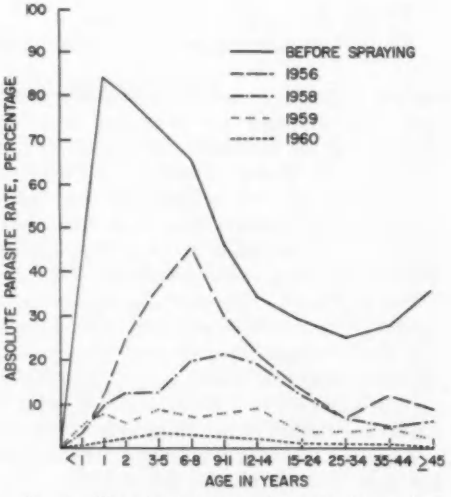

FIG. 2. The absolute parasite rate for various age groups before spraying and in the period 1956 to 1960.

is rather curious. The first peak in the curves corresponds to palpable spleens, not due to malaria. These aspecific enlarged spleens disappeared from children 2 years of age. After that age,

TABLE 3

Ratio of species of malaria parasites, per year, in Sentani

Survey	Per cent *P. falciparum*	Per cent *P. vivax*	Per cent *P. malariae*
1953, before spraying.....	43	22	35
1955, 1 year after spraying...	27	16	57
1956, 2 years after spraying..	39	22	39
1957, 3 years after spraying..	36	15	49
1958, 4 years after spraying..	41	15	44
1959,* 5 years after spraying.	75	20	5
1960,† 6 years after spraying.	48	52	—

* 4 months after 4th mass drug distribution.
† 3 months after 6th mass drug distribution.

TABLE 4

Spleen rates in the Sentani pilot area and in the Nimboran Valley before spraying and several years after spraying started

Age groups (in years)	Sentani				Nimboran		
	Before spraying	1956	1958	1960	Before spraying	1958	1960
Under 1	53	61	48	45	85	64	47
1	88	32	20	11	96	23	25
2	90	36	11	8	91	29	13
3–5	97	68	33	15	99	49	39
6–8	99	94	62	26	98	85	66
9–11	99	92	79	49	99	86	73
12–14	99	93	79	45	99	82	89
15–24	88	83	65	43	88	90	71
25–34	90	83	70	54	90	89	69
35–44	84	82	71	53	84	88	66
Over 44	82	74	55	47	82	82	49
Average.....	90	77	54	32	93	83	59

malaria was responsible for the palpable spleens. It appears that, if transmission is interrupted, spleens in children under the age of 10 years retract more easily below the coastal margin than do the scarred spleens of older people. Many Papuan spleens remain palpable for years after the infections are cured. Before spraying, the "saddle" phenomenon was obscured because, by the high number of infections at an early age, the peak of the aspecific spleens merged in that of the malarial spleens.

Possible causes of the aspecific spleen enlargement in infants were discussed elsewhere.[2] The

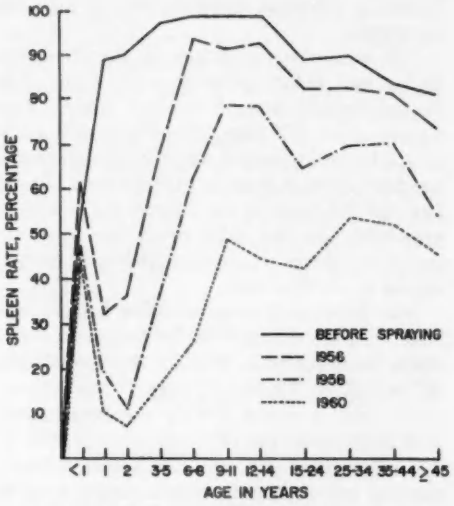

FIG. 3. Spleen rates observed in the various age groups before spraying and during 1956, 1958 and 1960.

minimum spleen rate at the age of 2 years, some years after spraying, is also in evidence in the Nimboran children (Table 4).

In Sentani a steady decline to a very low figure in the absolute parasite rates is seen, but in the Nimboran Valley it appears that the decline stopped after the 1959 survey (Table 2). In Sentani more drugs were distributed, but the Nimboran villages were sprayed more frequently. In Sentani, eradication seems possible by the methods used so far, but in the Nimboran Valley this possibility appears to be a remote one. Both areas are regarded as holoendemic.

However, circumstances in Sentani are more favorable from the point of view of malaria control. Experience has taught us, when causes for success or failure of malaria eradication measures are considered, to look at one basic and at several secondary factors. The basic factor is the number of infections to which, on an average, members of the population of an area are exposed before control measures are begun. Secondary factors are the type and quality of houses; the habit of sleeping inside the houses, on the gallery, or in shelters in gardens and sago marshes; nomadism; state of nutrition; etc. If the transmission rate is high, the secondary factors must be favorable for eradication to be achieved. If any one is un-

favorable, attempts at eradication may be disappointing.

The number of infections in the Nimboran Valley was, before spraying, much higher than in the Sentani area. Secondary factors were almost comparable except that the state of nutrition is better in Sentani, while Nimboran people are more prone to sleep in garden houses. Therefore, the difference in the basic factor is mainly responsible for the difference between almost complete success in Sentani and only partial success in the Nimboran.

Other holoendemic areas. Of other holoendemic areas, only one example will be discussed briefly: this is the Sekoli plain, south of the Sentani area and east of the Nimboran Valley. It was not surveyed before spraying, but the number of infections must have been of the same magnitude as that in the Nimboran area. The history of house spraying and of drug distribution is the same as that of the Nimboran Valley. However, houses are poorer and people have a habit of sleeping more often in garden houses. In 1957, the absolute parasite rate was still 34%; in 1959 and 1960 it was 33% and 36%, respectively. This illustrates the effect of strongly counteracting secondary factors.

Moderately endemic areas. Housing is seldom ideal in New Guinea, and some outdoor sleeping usually occurs everywhere. Nevertheless, in moderately endemic areas, unfavorable secondary factors may not always endanger the outcome of our efforts.

In the Schouten Islands, malaria was practically eradicated. Before spraying, parasite rates were between 20% and 30%. Spraying started in 1955. In 1959, after three drug distributions, only 60 positives in 23,006 slides (0.25%) could be detected.

In Japen and in the Wandamen Bay area the degree of endemicity before spraying varied between meso- and hyperendemic. Early in 1960, after five drug distributions, the average parasite rate in Japen was 4.6%, and in the Wandamen Bay area 1.9%. The somewhat higher figure in Japen is the result of surprisingly high rates in a few small villages.

Control measures in the Radja Ampat Islands, to the west of New Guinea, are promising. Only two spraying rounds and two drug distributions have been completed so far, but by early 1960 in South Waigeo there was a drop in parasite rates from 16% before spraying to 4.3% 1 year after spraying.

Even in moderately endemic areas success is not always assured. This is seen in results from the coastal area near Manokwari. People live alternately in two villages: one at altitudes up to 1,600 meters in the Arfak Mountains, the other one along the coast. There is very little or no transmission above 1,000 meters. However, on the valley floors anophelines bite frequently. Here, people sleep in bivouacs on their many trips. After 4 years of spraying and three drug distributions, the absolute parasite rate in a group of coastal villages was even higher (46%) than before control measures were started (28%). Drug distributions, therefore, were stopped and spraying recently was extended to the mountain villages up to 1,000 meters.

In Merauke seasonal malaria prevails; this is more easily controlled. In a few coastal villages the absolute parasite rates dropped from 31% before spraying, to 3% before drug distributing, and then to 1% early in 1960, after two drug distributions.

DISCUSSION

From the above observations it may be concluded that in New Guinea eradication cannot always be achieved with the methods used so far. Drug distributions appeared to be of great help in bringing down the parasite rates more rapidly, but only in areas where these rates already showed a tendency to diminish because of residual house spraying. In other words, they were effective where the reproduction rate[19] was below one.

Objections might be made to the interval of 3 months between the resurvey and the last drug distribution. This period, however, is a minimum. On many occasions it was longer, *e.g.*, in the Schouten Islands. Here, the majority of residual infections were *P. malariae*, presumably relapses. Some relapses of this parasite can be expected in the Sentani and Nimboran areas also. It is not likely, however, that they will greatly influence the parasite rates. This conclusion is partly based upon the assumption that immunity of the population is still sufficiently strong to be of help in bringing down the rates. The opinion of Mackerras and Sandars[20] that Papuans may possess an innate resistance to *P. falciparum* is supported by some of our observations.[16]

The point of eradication has practically been reached in the Schouten Islands, and is within the realm of possibility in the Wandamen Bay area, around Merauke, in most of the Radja Ampat Islands, in Japen—with some special attention for the few villages with high rates—and even in holoendemic Sentani. The results of extension of spraying operations in the area near Manokwari are awaited. However, for holoendemic areas, inhabited by people of a roving disposition, it seems apparent that measures other than spraying are necessary.

Two possibilities may be considered. The first, education of the people accompanied by economic development, takes time. Recently, small farms have been given to selected Papuans, to be cultivated under supervision of the Agricultural Service. An agricultural development project, including drainage works, is being considered for the Sekoli plain.

The second possibility is Pinotti's[21] method of distributing medicated salt. Experiments in the holoendemic Arso and Waris districts have produced promising preliminary results, in that *P. vivax* and *P. malariae* have practically disappeared after 3 months of pyrimethaminized salt distribution. Unfortunately, *P. falciparum* developed resistance to the drug, but it is hoped that this parasite will disappear also, as soon as chloroquine has been mixed with the salt.[22]

There are still areas in Netherlands New Guinea for which residual spraying combined with drug distribution is a promising method. They will be gradually covered in the next few years. For the problem areas, our hopes for the near future are founded on medicated salt.

SUMMARY

In a campaign of 7 years' duration to control malaria in Netherlands New Guinea, house spraying was combined with mass distributions of chloroquine and pyrimethamine.

In several moderately endemic areas, eradication was practically or almost achieved. Even in a holoendemic region, a point has been reached where eradication seems possible. However, in other areas, where people are of a roving disposition, results of house spraying and drug distribution were disappointing. The number of infections to which a population is exposed before control measures come into operation appears to be of major importance in relation to success or failure of eradication efforts. Aspects of holoendemic malaria, on which a new classification of malaria endemicity is based, are discussed. Changes in the ratio of the species of malaria parasites were not in accordance with expectations, *falciparum* showing an unexpected resurgence attributed to a more rapid loss of immunity to this species than to *vivax*. Future policy will be to continue residual spraying and drug distribution, supplemented by the use of medicated salt in problem areas and gradual bonification.

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LABORATORY STUDIES ON 3-CHLORO-9-(4-DIETHYLAMINO-1-METHYLBUTYLAMINO) ACRIDINE,10-OXIDE DIHYDROCHLORIDE (CI-423) AS AN ANTIMALARIAL COMPOUND

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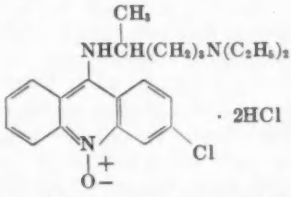
Although a large number of acridines have been examined for antimalarial activity,[1] no acridine has been shown to be a better antimalarial drug than quinacrine. Recent chemical work in these laboratories[2] on a series of acridine N-oxides has provided a new compound, CI-423, that is more potent than quinacrine against *Plasmodium cynomolgi* in monkeys and *P. lophurae* in chicks; furthermore, in contrast to quinacrine, the new compound has not caused visible staining of the skin during relatively prolonged administration to rodents. Data bearing on these properties of CI-423 form the substance of this report.

MATERIALS AND METHODS

Drugs. CI-423 is a bitter, water-soluble, yellow solid that contains 84.1% active base; it is identified chemically by the following structure and name.



3-chloro-9-(4-diethylamino-1-methylbutylamino)acridine, 10-oxide, dihydrochloride

Amodiaquin, quinacrine, and quinine were used as reference drugs; the first two were in the usual form of the dihydrochloride dihydrate salts and the latter as the dihydrochloride salt.

All drug dosages are expressed in terms of the free base.

Avian malaria assays. P. lophurae (12A strain) infections in chicks were used for assaying suppressive antimalarial activity as in previous work;[3, 4] hence only salient features of the method need to be reviewed here. White Leghorn chicks were placed on medicated diets when approximately 8 days old; the following day each was infected with 1×10^9 blood parasites/kg of body weight; 4 days later (5th day of medication) Giemsa-stained blood smears were prepared; the parasitemia was determined in terms of the percentage of cells parasitized. The geometric mean percentage of cells parasitized in a treated group in relation to that in a parallel untreated group served as the basis for estimating activity.

Simian malaria studies. Trophozoite-induced infections of *P. cynomolgi*† in rhesus monkeys were used. Infections were induced by the intravenous injection of 0.5×10^6 parasites. The efficacy of treatment was determined from the immediate course of the parasitemia and from the indications of whether or not the infections were cured.

Both thick and thin Giemsa-stained blood smears were prepared from a marginal ear vein, but only thin smears were used for quantitative estimates of the parasitemia. The following procedure was used in determining the degree of parasitemia, expressed as parasites/10,000 erythrocytes. Approximately 25 Howard disc fields (under × 930 magnification and containing 100 to 200 erythrocytes per field) were first scanned for a preliminary estimate of the parasite density. Parasites were then counted in 100 fields when there were less than 2 per field, in 25 fields when there were 2 to 5 per field, and in 10 fields when there were more than 5 per field. The number of erythrocytes per field was estimated from subsquares as in hemocytometer counting. This procedure, which permitted a highly accurate count, was confirmed by many replicate determinations for all except the lowest parasite

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† We are indebted to Dr. Frederick Coulston, Sterling-Winthrop Research Institute, Rensselaer, New York, for supplying us with this strain after he had procured it from Dr. L. H. Schmidt, Christ Hospital, Institute of Medical Research, Cincinnati.

densities. Sufficient cells were examined for most of the latter so that the probable error did not exceed 10%.[5]

Whether infections in treated monkeys remained latent or were cured was determined by a slight modification of the procedures of Schmidt *et al.*[6] Blood smears were examined daily for approximately 1 month after treatment; the spleen was then removed, and blood smears were examined almost daily for an additional month. Schmidt *et al.*[6] found splenectomy to be a powerful relapse provocative, and a demonstration of its efficacy was observed in one latent untreated control infection in this study.

Treatment consisted of either one or seven doses of the drug by stomach tube. The individual doses were dissolved in 15 ml of water. Treatment of initial infections was started on the 7th day after the injection of parasites, at which time there was an ascending parasitemia. Recrudescences were treated with appropriate precaution for distinguishing between the effects of treatment and of acquired immunity. The background and interpretation of the treatment of recrudescences are considered more fully in the section on results.

TABLE 1

Suppressive effects of CI-423 and of quinacrine against Plasmodium lophurae *in chicks**

Drug	Per cent drug in diet	Drug intake mg/kg/day	Per cent cells parasitized†	No. birds	Exp. no.
CI-423	0.00313	—	6.5	3	320
	0.025	43	0.04	3	317
	0.1	115	0.01	3	317
CI-423	0.00156	3	37.0	6	333
	0.00313	5	3.0	6	333
	0.00625	11	0.16	6	333
	0.0125	22	<0.02	6	333
Quinacrine	0.00625	11	30.6	6	333
	0.0125	23	0.8	6	333
	0.025	45	0.08	6	333
Untreated controls			39.1	6	317
			29.3	6	320
			32.5	6	333

* Drugs were given in the diet for 5 days, starting the day before infection.

† On the 4th day after infection.

RESULTS

Suppression of P. lophurae *infections in chicks.* Data depicting the effects of CI-423 and of quinacrine are presented in Table 1. The effects of quinine diet concentrations of 0.03 and 0.05% were determined in each experiment, with groups of 3 chicks used at each level, to provide a continuing check on the response of the infection to a reference drug. These results are not tabulated since they are in agreement with previous, more extensive data[4, 7] that indicate the ED_{50} of quinine to be approximately 46 to 59 mg/kg/day. CI-423 was effective proportional to the amount ingested and may be seen from the tabulated data to have an ED_{50} of approximately 4 mg/kg/day. The data from experiment 333 and from other unpublished tests indicate the ED_{50} of quinacrine to be about 16 mg/kg/day. Previous similar testing has shown amodiaquin to be about 30 times as active as quinine.[3] Thus, in drug-diet tests against *P. lophurae* in chicks, CI-423 is of the order of 12 to 15 times as active as quinine, 4 times as active as quinacrine, and one-half as active as amodiaquin.

Untreated P. cynomolgi *infections in rhesus monkeys.* The courses of untreated infections, as manifested by almost daily blood smears, were followed usually to the first remission in 8 untreated animals and to the 7th day after infection in 29 others that were treated subsequently. Although all animals within an experiment were infected from a common pool of parasites, circumstances precluded passage of the strain in the probable optimal manner for inducing highly uniform infections among the 8 experiments conducted. Other likely sources of variability were differences in age, weight, general condition, and sex of the hosts. Nevertheless, the infections conformed to a rather consistent pattern. Data on the 8 control monkeys are summarized in Table 2. The periods to patency (10,000 erythrocyte examinations) ranged from 4 to 7 days; the parasitemias reached peaks of 690 to 1,900 parasites and occurred on the 9th to the 18th day after infection; and the first remissions occurred 20 to 34 days after infection. The sum of the daily parasite counts during the entire period of patency—"the elapsed infection"—ranged from 2,133 to 7,441. The 29 other monkeys all developed patent infections within 7 days and on an average within 4.5 days. However, the 7th day parasitemia within experiments varied as

TABLE 2

Summary of untreated control monkeys

Exp. no.	Monkey			Parasitemia*			Days after infection to		
	No.	Sex	Wt. (kg)	7th day	Peak	Total†	Patency‡	Peak parasitemia	1st remission§
1	B-140	Male	2.95	6	1040	3282	5	12	32
2	B-371	Male	3.35	3	769	2133	7	18	32
3	B-254	Female	3.64	117	1040	3666	4	9	24
4	B-668	Male	3.21	39	910	>3056	4	10	>31
5	B-966	Male	2.24	36	1900	7441	4	12	20
6	B-975	Male	2.81	80	690	4685	4	15	23
7	B-368	Female	3.21	142	840	3647	5¶	10	34‖
8	B-737	Female	1.85	229	1400	4830	5	9	32

* Expressed in parasites/10,000 RBC.

† Index of elapsed infection: sum of daily parasite counts (in parasites/10,000 RBC) during entire period of patency up to 1st remission.

‡ Days to 1st positive blood smear: 10,000 RBC examined.

§ Days to last positive blood smear: 10,000 RBC examined.

‖ Splenectomy on 61st day after infection provoked a rapid and severe relapse characterized by a peak parasitemia of 4,100 parasites/10,000 RBC on 4th post-operative day.

¶ 5 or less.

greatly as between experiments (see the fifth column of Tables 2, 3, and 4), presumably a consequence of host heterogeneity.

Splenectomy has been amply demonstrated to be a highly dependable means of provoking relapses in rhesus monkeys harboring latent infections of the present strain of *P. cynomolgi*.[6] Hence, the observation of this effect in a single untreated animal (B-368, Table 2) sufficed to confirm the relapse-enhancing effect of splenectomy under the conditions of this study. The sequence of events after the injection of parasitized blood in monkey B-368 was as follows: The initial patent infection lasted from about the 5th through the 33rd day; the blood was either negative or had very few parasites from the 34th through the 61st day; the spleen was removed on the 62nd day; and parasitemias of 192, 4,100, 2,510, and 1,933 occurred, respectively, on the 64th, 66th, 67th, and 68th days.

Effects of CI-423 against P. cynomolgi *in monkeys and comparisons with amodiaquin and quinacrine.* The work was initiated on the premise that the rate and duration of parasitemia suppression, relative to drug dose, would serve as the main bases for appraising therapeutic activity and that radical cure would not be an important criterion. The multiple dose amounts of amodiaquin and quinacrine used for parasitemia suppression were suggested by the work of others.[6, 8, 9] The unexpected potency of CI-423 eventually led, however, to considerable use of the radical cure criterion.

Data depicting the over-all effects of the three drugs when given orally either once daily for 7 consecutive days or as a single dose are summarized in Table 3. These data show that the efficacy of the lowest dose of each drug varied according to the severity of infection. Such variability, which might be expected at threshold dose levels, gave assurance that the range of doses was appropriate for the intended comparisons. Hence, the validity of the conclusions drawn seems to be limited only by the number of replications in the testing.

Perusal of the multiple dose data shows that CI-423 suppressed the parasitemia in all of seven monkeys given doses ranging from 1.25 to 5.0 mg/kg/day. The rate and duration of suppression was directly proportional to the dose, if appropriate allowance is made for the variations in infection severity. Doses of 2.5 or 5.0 mg/kg/day rapidly suppressed the acute parasitemia and cured the animals.

Amodiaquin was given on the multiple dose schedule to three monkeys. Doses of 1.25 or 2.5 mg/kg/day checked the ascending parasitemia, but they failed to clear the blood of parasites.

TABLE 3

Comparative effects of CI-423, amodiaquin, and quinacrine when the drugs were given orally once daily for 7 consecutive days or in a single dose

Drug	Mg/kg daily	Pre-treatment				Days to become negative	Days remained negative	Monkey no.	Exp. no.
		Days inf.	Stage of infection	Parasitemia*					
				Just before treatment	Total elapsed†				
7 daily doses									
CI-423	1.25	7	Initial	146	181	6	1	B-965	5
	1.25	7	Initial	1	1	3	9	B-967	6
	1.25	7	Initial	160	231	7	20	B-641	7
	2.5	7	Initial	288	328	2	>82 RC‡	B-312	4
	2.5	7	Initial	107	128	3	>53 RC	B-972	6
	2.5	7	Initial	163	211	3	>83 RC	B-638	7
	5.0	7	Initial	236	307	1	>85 RC	B-511	7
Amodiaquin	1.25	7	Initial	253	343	∞§	0	B-250	7
	2.5	7	Initial	149	194	∞	0	B-646	7
	5.0	7	Initial	125	176	1	19	B-615	7
Quinacrine	1.25	7	Initial	175	201	∞	0	B-249	3
	2.5	7	Initial	5	6	1	11	B-370	2
	2.5	7	Initial	533	595	∞	0	A-986	4
	5.0	7	Initial	5	7	2	15	B-155	1
	5.0	7	Initial	159	180	3	15	B-971	6
	5.0	7	Initial	112	118	3	12	B-968	5
Single dose									
CI-423	5	18	1st recrud.	6	123	1	10	D-765	8
	5	15	1st recrud.	880	1371	1	9	D-710	8
	10	7	Initial	238	310	2	10	D-736	8
	10	21	1st recrud.	10	326	1	11	D-736	8
	10	27	1st recrud.	6	127	1	17	B-974	8
	10	27	2nd recrud.	84	1469	2	12	D-710	8
	20	7	Initial	96	136	2	>62 RC	D-747	8
	20	36	2nd recrud.	33	373	1	>65 RC	D-736	8
	20	42	3rd recrud.	3	1476	2	>59 RC	D-710	8
	20	49	3rd recrud.	7	139	1	>56 RC	D-765	8
	40	7	Initial	227	276	2	>62 RC	D-731	8
Amodiaquin	10	7	Initial	111	139	5	1	D-710	8
	10	46	2nd recrud.	1	130	∞	0	B-974	8
	20	7	Initial	88	112	2	8	D-765	8
	20	49	3rd recrud.	19	179	2	9	B-974	8
	40	7	Initial	81	119	2	15	B-974	8
	40	30	2nd recrud.	1	125	1	16	D-765	8
	80	63	4th recrud.	6	187	1	51 RC‖	B-974	8

* See footnotes Table 2.

† See footnotes Table 2.

‡ RC, radical cure. Based on negative blood smears for the indicated number of days including *26* to *55* days of post-splenectomy examination.

§ ∞, did not become negative as result of treatment.

‖ Followed only 2 weeks after splenectomy, owing to intercurrent infection.

Doses of 5.0 mg/kg/day rapidly terminated the patent parasitemia, and prevented a recrudescence for 19 days (13 days after the end of treatment).

Six monkeys were treated with multiple doses of quinacrine. Doses of 1.25 mg/kg/day had no discernible effect on the parasitemia, which increased 8-fold during treatment (monkey B-249) and simulated closely the course of infection in the untreated control (monkey B-254, Table 2). Doses of 2.5 mg/kg/day were ineffective against a severe infection (monkey A-986) but completely suppressed a mild infection (monkey B-370) for 11 days (5 days after the end of treatment). Doses of 5.0 mg/kg/day terminated the patent parasitemia within 3 days, but permitted recrudescences within 12 to 15 days (8 to 11 days after the end of treatment).

Comparison of the three drugs, taking all criteria into account, suggests CI-423 to be more than twice as active as amodiaquin and quinacrine. CI-423 was significantly superior to quinacrine in conferring radical cure ($P = 0.05$[10]). The data on amodiaquin are insufficient for statistical analysis.

Before the effects of single dose treatment are reviewed, explanation is needed regarding the use of recrudescences for appraising activity. It was noted early in the course of this study that a suppressive but subcurative level of drug administered prior to the acquisition of immunity, as manifested by a declining parasitemia, simply deferred the course of the acute infection. This observation suggested that the same animal could be retreated if it had not experienced enough infection to induce a high degree of immunity and if one could risk the likelihood of inducing drug-resistant parasites. The entity "total elapsed parasitemia" was adopted as an appropriate index of the status of immunity, and was computed by adding together the daily parasite counts throughout the patent period. The likelihood that drug-resistant strains would arise with the types of drugs used in this work was deemed to be negligible, since all attempts to induce amodiaquin- or quinacrine-resistant strains have been unsuccessful (cf. Thompson,[11] for a review of the literature).

CI-423 in a single dose of 5 to 40 mg/kg consistently terminated the patent parasitemia and was tolerated well in a total of 11 trials; 5 or 10 mg/kg, given to 6 monkeys, permitted recrudescences after intervals of 9 to 17 days; 20 or 40 mg/kg conferred radical cure in each of 5 animals.

Amodiaquin was tested as a single dose on 7 occasions. A dose of 10 mg/kg only temporarily inhibited the progress of the parasitemia. With 20 or 40 mg/kg the parasitemia was completely suppressed but recrudescence ensued in each instance within 8 to 16 days. A dose of 80 mg/kg rapidly controlled the parasitemia and appeared to confer cure.

The two drugs were tested under quite comparable conditions with regard to the severity and stage of infection, and review of the single dose data indicates CI-423 to be two to four times as potent as amodiaquin. The difference in their radical curative effects is significant at the 5% level.[10] Moreover, the period of remission was about the same for 5 mg/kg of CI-423 as for 20 mg/kg of amodiaquin, and only slightly less for 10 mg/kg of CI-423 than for 40 mg/kg of amodiaquin.

A comparison of the rate of action and of parasite damage by CI-423 and amodiaquin was made in 7 monkeys infected from a common pool of parasites (Table 4). One monkey was observed as an untreated control and the others were so treated as to enable comparison of the drugs in a single oral dose of 40, 20, and 10 mg/kg. Blood smears were prepared just before treatment and at 6, 12, 18, 24, 28, 46, and 72 hours after treatment. Both drugs acted rapidly and at about the same rate, as reflected by direct damage to the parasites and a rapid reduction of the parasitemia. The effects of each drug were detectable within 6 hours and were strongly evident within 24 hours.

CI-423 and amodiaquin caused similar changes in the parasites and they affected all circulating stages. The effects were progressive with dosage and time. They caused the cytoplasm of asexual stages to stain a more dark grayish-blue than normally and interfered with pigment formation. Neither drug seemed to induce a specific change in the appearance of the chromatin, but each inhibited the growth of asexual stages. The ring stages frequently appeared as bands with no visible connection between the chromatin and cytoplasm. Owing to the time of treatment and inhibition of asexual growth, very few large trophozoites and schizonts were available for study. Those that were seen were almost devoid

TABLE 4

Comparison of rate of action by CI-423 and amodiaquin after a single oral dose (all monkeys infected from a common pool of parasites; treatment given 7 days after infection)

Hours after treatment	Parasites/10,000 RBC						
	CI-423 (mg/kg)			Amodiaquin (mg/kg)			Untreated control
	10	20	40	10	20	40	
0	238	96	227	111	88	81	229
6	192*	62*	77*	189*	64*	64*	288
12	44*	15*	15*	19*	5*	8*	340
18	9*	4*	3*	5*	2*	4*	627
24	1.4*	0	0.7*	1.4*	0.5*	1.4*	576
28	0.7*	0.7*	0.7*	0.7*	1.4*	0.7*	612
46	0	0	0	0	0	0	1400
72	0	0	0	1	0	0	1230
Identity of monkeys							
Wt. (kg)	1.75	1.95	1.7	1.7	1.6	3.0	1.9
No.	D-736	D-747	D-731	D-710	D-765	D-974	D-737
Sex	Male	Female	Male	Female	Male		Female

* Parasites damaged.

of pigment. Both macro- and microgametocytes were damaged, the cytoplasm stained abnormally, and the pigment was scanty and appeared as a single mass or as a few granules in a vacuole. Although the "bronze effect" of amodiaquin on *P. lophurae* pigment[11] could not be detected in *P. cynomolgi*, it is noteworthy that this drug affected pigment formation in both of these parasites.

Tests for staining of skin by CI-423 in mice and rats. Inasmuch as one of the more frequent objections to quinacrine is staining of the skin and sclera, it was deemed worthwhile to determine if CI-423 also had this liability. Accordingly, CI-423 and quinacrine were studied in adult albino mice and rats (153 to 173 g weight range).

Three groups of 5 mice each were used; one group was sham-dosed with water and the other two groups were given, respectively, CI-423 and quinacrine by stomach tube in doses of 50 mg/kg twice daily for 15 consecutive days. All mice survived. No staining of the cutaneous surfaces could be detected in the CI-423 mice either by examining the intact animal or the under surface of the skin under natural and ultraviolet light. In contrast, quinacrine stained the skin and internal organs intensely.

For the tests in rats 4 groups of 10 were fed, respectively, CI-423 diet concentrations of 0.05 and 0.1% and quinacrine diet concentrations of 0.03 and 0.06% for 4 weeks.* As in mice, no staining was detected in the skin of the CI-423 rats, although the quinacrine rats showed intense staining. The internal organs of the CI-423 rats also appeared to be of normal color before the vascular system was perfused with physiologic saline in preparation for formalin fixation by the vascular route. Following perfusion, a faint yellow tinge could be detected in the lungs, liver, pancreas, salivary gland, thymus, and the kidneys but not in the skin or other organs. Sections of the kidney showed deep staining in the medulla only. Such staining of the organs showed that the drug is absorbed from the gastro-intestinal tract of rats. The rodent tests suggest that CI-423 should not be cosmetically objectionable because of obvious staining.

DISCUSSION

The animal infections used here have proved in the past to be useful means of selecting drugs for trial in human malaria. Hence, the high efficacy of CI-423 in animals, relative to drugs of known human value, indicates that it is likely to have good suppressive activity in man; but

* The studies in rats were conducted by Dr. J. K. Weston, Dr. K. Weston, and associates in these laboratories.

neither the animal data nor chemorphological considerations provide a basis for predicting that it would have high radical curative activity against relapsing vivax malaria. Judging from the periods of remission in monkeys following treatment with subcurative doses, CI-423 is dissipated at about the same rate as amodiaquin. This indication, coupled with higher potency, suggests that CI-423 may be superior to amodiaquin and quinacrine either in controlling the symptoms of malaria at lower doses or in giving longer remissions at equivalent doses. Either outcome would, of course, represent a distinct therapeutic advance. CI-423 has the further advantage over quinacrine of being unlikely to stain the skin.

Formal pre-clinical toxicity studies in animals[12] and observations on the tolerance of the drug by human volunteers[13] have indicated that CI-423 is tolerated sufficiently well for trial in the treatment of human malaria.

This study points to a method for performing several tests for suppressive action against *P. cynomolgi* in the same monkey. However, the method is only applicable for drugs that are unlikely to induce resistance. Its chief features are standardized infection procedures, timing of treatment so the animal does not experience enough parasitemia for the acquisition of an effective immunity, and a running estimate of the status of immunity. For the latter, the previously-defined "total elapsed parasitemia" is used. The initial treatment is applied early in the course of the ascending initial parasitemia and retreatment is instituted at a comparable time in the recrudescences. The usefulness of the procedure is demonstrated by the single dose data on CI-423 and amodiaquin (see Table 3). Thus, retreatment through 3 recrudescences during a 2-month period gave results, relative to potency, rate of action, and duration of effect, similar to those of the initial treatment tests. Theoretically, one would anticipate that a basis for delineating between the effects of drug and of acquired immunity would progressively diminish as the "total elapsed parasitemia" in the treated animal approached that in the untreated control. Experience so far indicates that this discernment can be made satisfactorily as long as the treated value is less than one-half the control value.

SUMMARY

This study deals with the compound CI-423 [3-chloro-9(4-diethylamino-1-methylbutylamino) acridine, 10-oxide, dihydrochloride] as a potential non-staining, suppressive antimalarial drug.

Comparative testing by the drug-diet method against trophozoite-induced *Plasmodium lophurae* infections in chicks indicated CI-423 to be approximately 16 times as active as quinine, 4 times as active as quinacrine, and one-half as active as amodiaquin.

Comparisons on a single or multiple oral dose basis against trophozoite-induced *Plasmodium cynomolgi* infections in rhesus monkeys showed CI-423 to be distinctly more potent than either amodiaquin or quinacrine in rapidly suppressing the acute parasitemia and curing the animals.

In contrast to quinacrine, CI-423 did not induce visible staining of the skin of mice or rats when given for respective periods of 2 and 4 weeks.

ACKNOWLEDGMENTS

The authors are indebted to Dr. E. F. Elslager and Mr. F. H. Tendick for supplies of the drugs, and to Dr. A. C. Bratton, Jr. for encouragement and advice.

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HUMAN ISOSPOROSIS IN THE WESTERN HEMISPHERE

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Isosporosis is infection with species of the coccidian genus *Isospora*, of which *I. belli* and *I. hominis* are the human representatives. These two protozoa are believed to parasitize the mucosa and at times the submucosa of the intestinal wall, although it is not known at what level they inhabit the intestine[1–3] since autopsy evidence is lacking. As a result of experimental infection of two Japanese physicians[4] and a presumptive accidental laboratory exposure in the United States[5] there is substantial evidence that the mature oöcyst of *I. belli* is the infective stage of this species. About 10 days after ingestion of these oöcysts immature oöcysts of the next generation begin to appear in the subject's stools. If one may compare infections of *I. belli* and *I. hominis* with those produced by the closely related species *I. bigemina* var. *canis* in the dog and *I. felis* in the cat,[6, 7] following ingestion of the ripe oöcysts by man the enclosed sporozoites escape from their sporocyst walls in the upper part of the small intestine and initiate infection by entering the mucosa at a somewhat lower level. Here they transform into trophozoites, each of which gives rise internally to a relatively large number of merozoites. The latter escape from the infected host cells and enter other mucosal cells, in which they produce additional asexual progeny. Later certain merozoites metamorphose into male and female gametocytes which produce the mature sex cells (gametes). Fertilization of female by male gametes results in the formation of the immature oöcysts, the stage which is evacuated in the stools.

GEOGRAPHICAL DISTRIBUTION AND SPECIES DIFFERENTIATION

Human isosporosis is essentially cosmopolitan but, like amebiasis and giardiasis, it is more common in tropical and subtropical than in temperate climates. During World War I epidemics developed in the Near East and during World War II in the Southwest Pacific islands, in both instances among military forces which were foreign to those areas. Usually human isosporosis has been diagnosed as a sporadic parasitosis; yet there are centers in which these infections are moderately endemic, viz., Southwest Pacific islands, Natal (South Africa),[1] São Paulo (Brazil), Cali (Colombia) and Santiago (Chile).

Magath[8] published a summary of the diagnosed cases of human isosporosis up to 1935. Seventy-five per cent of the total number (200) originated in the Near East and the remainder consisted of scattered cases from the Far East, Africa and the Americas. Diagnosis was based on discovery of the immature oöcysts of *Isospora*, usually from unconcentrated fecal smears of diarrheic stools. With rare exceptions the organism was referred to as "*I. hominis*," although in 1923 Wenyon[7] had distinguished two species, *I. belli*, the more prevalent one, and *I. hominis*, a less frequent parasite of man. Until recently most diagnoses designated as "*I. hominis*" in the medical and parasitological literature should have been *I. belli*. Today the difference between these two species is well established, based on the oöcysts recovered from freshly evacuated patient's stools. Those of *I. hominis* (Figures 1–10) have already matured and both of the sporocysts, which have typically escaped from the parent membrane, contain four well developed sporozoites; whereas those of *I. belli* (Figures 11–13) are immature and ripen only after they have been evacuated in the feces.

During the quarter century since Magath's publication[8] much information has been accumulated concerning the distribution of human *Isospora* infections, especially in the Americas. Forty-three additional cases have been reported from the United States, mostly from the southeastern states. We have knowledge of the follow-

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TABLE 1

Cases of human isosporosis diagnosed in the Western Hemisphere

Country and subdivision	Authority and reference no.	*Isospora belli**	*Isospora hominis*	Total
United States of America		*38* (+*1*)†	*6*	*44* (+*1*)
Tennessee	Meleney *et al.* (50)	1		
California	Kessel (51)		1	
California	Markel (52)		2	
Georgia	Routh *et al.* (53)	1	3	
Florida	Beck *et al.* (54)	1		
Tennessee	Webster (55)	3		
S. Carolina	Jeffery (5)	4 (+1)†		
S. Carolina	Jeffery (56)	28		
Mexico		*11*		*11*
Federal District	Beltrán and Larenas (10)	1		
Federal District	Beltrán (9)	2		
Federal District	Biagi and Portilla (11)	8		
Federal District	Carillo-Cárdenas (12)			
Costa Rica	Ruiz and Trejos (13)	*7*		*7*
Cuba	Kourí and Basnuevo (14)	*1*		*1*
Puerto Rico	Maldonado (15)	*1*		*1*
Venezuela	Briceño-Rossi (16)	*1*		*1*
Colombia		*33*	*12*	*45*
Bogotá	Muñoz Rivas (18)	2		
Cartagena	Montaña (17)	1		
Cali	Present report	30	12	
Perú		*9*		*9*
Lima	Souza Peixoto (22)	1		
Rimac Valley	Gonzáles-Mugaburu (19)	3		
Cordilleras	Payne *et al.* (21)	4		
Lima	Gonzáles-Mugaburu (20)	1		
Chile		*more than 645*	*more than 645*	
Santiago	Neghme and Silva (26)	1		
Santiago	Balmaceda *et al.* (23)	11		
Santiago	Jarpa (24)	more than 200		
Santiago	Orrego *et al.* (2)	1		
Santiago	Jarpa (25)	433		
Argentina		*3*		*3*
Entre Ríos	Castex and Greenway (28)	1		
Entre Ríos	Castex *et al.* (29)	1		
Buenos Aires	Bacigalupo (27)	1		
Uruguay		*2*		*2*
Montevideo	Hormaeche (30)	1		
Montevideo	Osimani (31)	1		
Brazil		*44*	*22*	*66*
Rio de Janeiro	Pinto and Pacheco (46)	1		
São Paulo	Carini (33)	1		
Rio de Janeiro	Barros (32)	1		
São Paulo	Prado (47)	1		
Minas Gerais	Neves and Motta (42)	5		
Minas Gerais	Neves and Penna Sobrinho (43)	1		
Santos	Consentino (34)	1		
São Paulo	Meira and Savastano (40)	6	6	
São Paulo	Vasconcelos (49)	3		
São Paulo	Corrêa (35)	3	1	
São Paulo	Rotondi (48)	1		
São Paulo	Pasqualini (44)	1		
São Paulo	Meira and Corrêa (39)	13	15	
Salvador	Pessôa (45)	2		
São Paulo	Pessôa (45)	3		
Recife	Malufe Ribeiro and Simões Barbosa (37)	1		

* In this table the species name *Isospora belli* is used for the majority of cases previously referred to as "*I. hominis*" unless the author has specifically distinguished between the two species.

† The numeral "1" in parentheses under *Isospora belli* which follows the reference to Jeffery[5] refers to a presumptive accidental laboratory infection.

ing diagnosed cases from Latin America: Mexico, 11;[9–12] Costa Rica, 7;[13] Cuba, 1;[14] Puerto Rico, 1;[15] Venezuela, 1;[16] Colombia, 45 (present study);[17, 18] Perú, 9;[19–22] Chile, more than 645;[2, 23–26] Argentina, 3;[27–29] Uruguay, 2;[30, 31] and Brazil, 66.[32–49] Six of the persons diagnosed in the United States, 12 of those in Colombia and 22 in Brazil were parasitized by *I. hominis*. In Mexico, Costa Rica, Perú and Chile only *I. belli* has been discovered.

Up to the present time no less than 835 cases of human isosporosis have been reported from the Western Hemisphere (Table 1). In the Parasitology Laboratory of the Department of Preventive Medicine and Public Health, University of Valle, Cali, Colombia, 30 persons have been found infected with *I. belli* and 12 with *I. hominis* during the past 3.5 years. Only 3 of the 30 and 2 of the 12 were diagnosed from direct fecal films. In contrast, only one of the total was negative by zinc sulfate centrifugal flotation technic.

EPIDEMIOLOGICAL DATA

Although human isosporosis occurs in all age groups, in our experience, as in Brazil,[37] many of the infected persons have been under 10 years of age and several have been infants. Infection is more common in families which live under poor economic and sanitary conditions but 6 of our diagnoses in Cali were made from processing stools of individuals who enjoy a superior standard of living.

At times human isosporosis becomes chronic, especially in groups subject to the stress of war coupled with poor nutrition and an insanitary environment. The senior author observed 40 patients of this type among American troops in the Philippines during World War II.

In the present study in Cali, Colombia, oöcysts of *I. belli* and sporocysts of *I. hominis* have seldom been observed in the stools of patients for more than 2 successive days, suggesting that the infection is relatively transient in the human host in this area, *i.e.*, it is rapidly eliminated.

Magath[8] emphasized the infrequency of human cases of isosporosis and considered that other hosts must serve as reservoirs of the infection. Rarely a cat or a dog associated with human infection has been found parasitized with *I. belli* or *I. hominis*,[51, 55] but no systematic investigation has been conducted in domestic animals in communities where human isosporosis has been diagnosed. In Cali our group[57] examined 528 freshly passed stools of dogs, collected in the homes and on the nearby streets of ward Siloé, where most of our human cases have been discovered. The sampling was considered to be adequate for the approximately 2,000 dogs in this area. The incidence percentage of species of *Isospora* diagnosed from these specimens was as follows: *I. belli*, 0.37; *I. hominis*, 24.6; *I. bigemina* var. *canis*, 3.41; and *I. felis*, 0.57. Infection with *I. belli* and *I. hominis* appears to be about as unstable in these animals as in man. Cats in this area are very uncommon; no opportunity developed to examine their feces. Ninety-two domestic rats and mice trapped in the homes of infected individuals were studied. The only coccidia discovered were species of *Eimeria* in 63 animals and a single oöcyst of *I. belli* in a Norway rat (unpublished data). Thus no evidence has been obtained suggesting that dogs, cats and rodents are true reservoirs of human *Isospora* in our study area.

DISCUSSION

The information summarized in this paper shows that human isosporosis is present in many areas of Latin America and is found in several localities in the southeastern United States and in southern California. The isolated reports from many Latin American countries and the lack of diagnoses of *I. belli* and *I. hominis* in parasitological surveys in other Latin American countries probably do not represent the true situation. Failure to discover these coccidia may be due to one or more of the following difficulties: (1) inexperience in recognizing the stages of this organism occasionally present in direct fecal smears; (2) omission of a suitable technic for concentrating the cysts essentially free of fecal debris; and (3) inadequate sampling of the population under investigation. With reference to this last point it has been estimated that in Brazil[45] the percentage incidence of human isosporosis is 0.1; in Cali, 1.0; and in Santiago, Chile, 1.8. The only published report of high incidence of human isosporosis was that of an epidemic in a North American institution for mentally handicapped children, where the percentage of infection was 18.0 in the children and 41.0 in the employees.[56]

Although in São Paulo, Cali, and the state of

Georgia both *I. belli* and *I. hominis* have been diagnosed, in Santiago where a majority of all cases in the Americas have been discovered, as well as in other American communities, only *I. belli* has been identified.

Except for incidental investigations in California[51] and Georgia[53] and the more extensive study which we have conducted (Giraldo *et al.*[17] and unpublished data) no attempt appears to have been made to discover the two species of *Isospora* of man in associated domestic animals. The evidence obtained in Cali suggests that infection with *I. belli* and *I. hominis* in the dog is as unstable as in man, that cats are too few in the area to constitute reservoir hosts of these coccidia, and that the only natural infection with coccidia in domestic rodents is due to species of *Eimeria*. Therefore we conclude that these animals are not satisfactory reservoirs of the two species of *Isospora* which parasitize man in our vicinity. How then are these infections maintained in an area such as Cali? The only plausible explanation which we are able to suggest is based on our own experimental evidence of the resistance of the cystic stage of these coccidia to unfavorable environmental conditions (unpublished data). In an unsanitated area like ward Siloé ample opportunity is provided for persons and dogs to obtain viable cysts from fecally contaminated ground in and around the homes, as well as from soiled clothing and other fomites. In this respect isosporosis parallels amebiasis and giardiasis, although the coccidian cysts are much the more resistant to drying and putrefaction. Nevertheless, before our postulate can be accepted as epidemiologically adequate it will be necessary to carry out carefully controlled experimental studies. Moreover, before it is possible to explain the numerous unsolved questions in human isosporosis it will be necessary to elucidate the entire life cycle of the etiological agents and to determine the relationship of the different stages of the parasites to the tissues of the human intestine. Biopsy snips employing the Crosby technic should be helpful in carrying out both of these objectives.

SUMMARY

Isosporosis in man is due to infection with *Isospora belli* and *I. hominis*, of which the former is the more prevalent form. Differential diagnosis is easily made on discovering the immature oöcysts of *I. belli* and the mature sporocysts of *I. hominis* in freshly evacuated stools.

Until 1935 there were only 200 known cases of human isosporosis in the world. At present at least 835 authentic cases have been diagnosed in the Americas, including isolated infections from many localities and endemic foci in São Paulo, Cali and Santiago. Examination of thousands of human stools, employing satisfactory concentration technics in addition to direct fecal smears, has indicated that the percentage rates of isosporosis in these three endemic centers are approximately 0.1, 1.0 and 1.8, respectively.

Although human isosporosis may become chronic, much more frequently it is only a temporary unstable infection in man and the dog. The one plausible explanation which we are able to suggest for the maintenance of *I. belli* and *I. hominis* endemically in a community is based on the resistance of the cysts to unfavorable environmental conditions, thus affording ample opportunity for human exposure.

Greater competence in the recognition of the stages of *Isospora* in the feces, use of a satisfactory concentration technic, and examination of thousands of fecal specimens are required to provide an accurate picture of isosporosis in a community.

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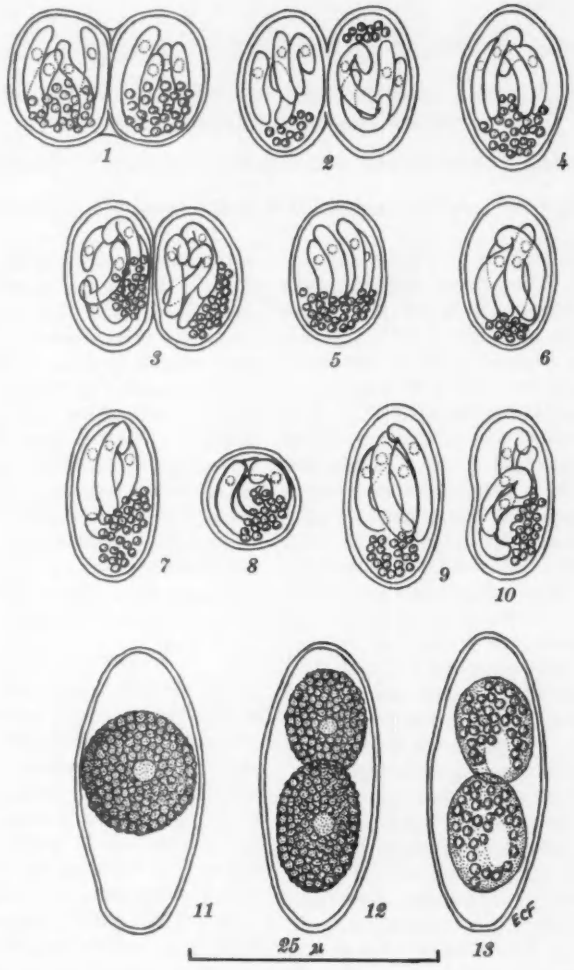

PLATE I

Differential characters of the two species of *Isospora* based on the stages seen in freshly passed feces. (Camera lucida drawings of stages recovered from human stools in Cali, Colombia.)

FIGS. 1–10. Mature sporocysts of *I. hominis*, freed of their oöcyst wall, each containing 4 sporozoites and residual granules. Figures 1 to 3 are twinned sporocysts. Figures 4 to 10 represent the more usual condition in which the sporocysts are separate. Figure 8 is a polar view.

FIGS. 11–13. Typical immature oöcysts of *I. belli*.

SEROLOGIC DIAGNOSIS OF *SCHISTOSOMA MANSONI* INFECTIONS

III. Isolation and Purification of Antigen from Adult *S. mansoni* for the Complement Fixation Test

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Fifty years ago Yoshimoto[1] demonstrated the fixation of complement by an extract of adult *Schistosoma japonicum* and homologous antiserum. Since that time numerous other methods of extracting antigen for complement fixation tests have been described. Relatively little attention[2-8] has been given to antigen purification, presumably because of the large quantities of worm material required. Recently, the development[9] of an apparatus for recovering schistosomes from the hepato-portal and mesenteric veins of infected laboratory animals so facilitated the collection of worms that an antigenic analysis based on physico-chemical methods was undertaken. The present report summarizes the findings.

MATERIALS AND METHODS

Source of adult schistosomes. Healthy young white mice (6 to 8 weeks) and hamsters (10 to 12 weeks) of both sexes were infected with approximately 200 and 500 *Schistosoma mansoni* cercariae, respectively. After 6 to 8 weeks the animals were killed and the worms collected by the Perf-O-Suction method.[9] The collected worms were washed in 0.9% NaCl solution until free of blood and tissue debris. The worms were pooled in a 10-ml Pyrex ampule (300 to 500 worms/ampule) and washed in two changes of distilled water. The contents of the ampule were lyophilized and the ampule was sealed hermetically under vacuum. As an additional precaution the worms were stored at −20°C until required for extraction.

Isolation of the lipid fractions. A 750-mg quantity of desiccated worms was ground 15 minutes with 20 ml of pre-chilled (−20°C) anhydrous ether‡ by a motor driven glass tissue grinder, the cylinder of which was immersed in a $CaCl_2$-ice-water bath (−15°C). The ethereal suspension was transferred quantitatively to a pre-chilled (−20°C) ultra fine fritted-glass filter§ fitted with a rubber stopper carrying a $CaCl_2$ drying tube. The filter was attached to the unchilled (25°C) 500-ml unit base containing 2 drops of freshly added unchilled ether. The entire unit was placed in the refrigerator (−20°C) where gaseous contraction within the flask drew the ether soluble substances of the worms through the filter. As the ether level fell more ether at −20°C was added until 200 ml had been used. The ether filtrate was then distilled under vacuum and the lipoidal residue (designated lipid fraction, L_1) was removed and placed in a vacuum desiccator at 3° to 6°C. The process of extracting with 200 ml of ether was repeated four times; the filtrates constituting lipid fractions L_2 to L_5 were distilled and placed in the desiccator.

Isolation of the protein fraction. The ether extracted worm residue was returned to the tissue grinder, where it was reground for 15 minutes in 20 ml of borate buffered saline solution (BBS)‖ at pH 8.0. During grinding, the cylinder of the grinder was immersed in an ice-water bath (0°C). The saline suspension was transferred to a beaker and diluted with BBS (0°C) to a volume of 75 ml, the dilution representing 10 mg of original worm material per ml of BBS. Extraction was allowed to proceed for 12 hours at 4°C. To facilitate extraction, the mixture was stirred constantly by an electric stirrer turning at 100 rpm. Centrifugation of the mixture for 30 minutes at 16,000 × G in the high speed head of the refrigerated centrifuge (4°C) yielded an insoluble dark brown residue and a clear brownish, viscous supernate (P_1). The residue was dried, weighed and discarded. After

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‡ Anhydrous ether, analytical reagent grade, Mallinckrodt Chemical Works, New York, Philadelphia, St. Louis.

§ Morton type, Arthur H. Thomas Co., Philadelphia.

‖ The buffer consisted of 12.405 g boric acid, 14.912 g potassium chloride in 1 liter of distilled water. For use 50.0 ml of this stock, plus 4.0 ml 0.2 M sodium hydroxide, was diluted to 200 ml with distilled water.

removing a 15-ml portion of P_1 for chemical, serological and electrophoretic analyses, the remainder (60 ml) was dialyzed with rotation for 12 hours in the cold (4°C) against 4 liters of distilled water. Centrifugation (30 min at 16,000 × G, 4°C) yielded a clear supernate (fraction P_2 alpha), and a white precipitate (P_2 beta) which was redissolved in 60 ml of BBS. P_2 alpha was desiccated from the frozen state and its volume restored to 60 ml with BBS. After 15 ml of P_2 alpha was removed for analyses the remainder (45 ml) was subjected to isoelectric precipitation by adjusting the pH to 4.8 with 0.1N HCl. The method employed was essentially that of Melcher.[10] The white precipitate that formed at pH 4.8 was removed by centrifugation and easily redissolved in 45 ml of BBS (pH 8.0). This fraction was considered as the acid insoluble fraction (P_3 alpha). The acid soluble substances (pH 4.8) were designated P_3 beta. Fifteen-milliliter samples of each were reserved for analyses. P_3 alpha (30 ml) was further fractionated by salting out in the cold (4°C) at increasing concentrations of ammonium sulfate; saturated $(NH_4)_2 SO_4$ was added, dropwise, with constant stirring. Precipitates which appeared at approximately 30% and 60% saturation were recovered by centrifugation after standing 4 hours at 4°C, and each was redissolved in 30 ml of BBS; the first was designated P_4 and the second, P_5. The supernate (P_6) was freed of ammonium sulfate by prolonged dialysis in the cold. It was lyophilized and volume restored to 30 ml with BBS.

Isolation of carbohydrate fraction. Separate experiments were employed to isolate protein-free carbohydrates[10] from 200 mg of ground, desiccated schistosomes. A white precipitate that was easily dissolved in distilled water was formed. The washed carbohydrate solution gave a positive Molish reaction and a negative micro-Kjeldahl test for nitrogen. The final step was to again precipitate the carbohydrates with cold ethanol and redissolve the precipitate in 20 ml of BBS for serological testing.

Complement fixation procedure. Fractions isolated were tested as antigen* in a complement fixation test. The method used was essentially that of Kent *et al.*[11] and Kent.[12] This technic was first employed in schistosomiasis by Chaffee *et al.*[5] The single exception to the original technic was use of triethanolamine buffered saline[13] instead of veronal buffered saline. Antigen determinations were of the block type. Serial 2-fold dilutions of antigen were titrated against serial 2-fold dilutions of schistosomal antisera. In each instance, two titrations were performed: one with serum from an individual with early schistosomiasis (stool with eggs) and the other with serum from a monkey proven, by challenge, to be immune to the infection.[14] The block type titration permitted observance of maximal amounts of antigen obtained in a given fraction and facilitated comparison with the amount obtained in other fractions. An additional titration with serial 2-fold dilutions of syphilitic sera was performed with P_1 and all lipoidal fractions (L_1 to L_5); each fraction had been restored to 20 ml with ethanol.

Maximal antigenic activity for any fractionation study was expressed as ++++. The presence of less antigen in any fraction was designated, arbitrarily, as +++, ++, or +; no detectable antigen was expressed as 0.

Electrophoresis. Paper electrophoretic studies were performed with a Durrum type hanging strip cell.† Various buffers at different pH and different ionic strengths were employed at various voltages. The best resolution was obtained with a veronal buffer (VBS)‡ of pH 8.6 and ionic strength of 0.1 for 10 hours at 250 volts. Strips were stained with bromo-phenol blue for proteins or with fuchsin-Koiw for carbohydrates.

RESULTS

Although 750 mg of desiccated worms was used in each fractionation study, all results are expressed in terms of 100 mg to simplify presentation of data.

Lipoidal fractions. The dry weights of the ether soluble fractions (L_1 to L_5) were 12.0, 4.6, 5.4, 0.6 and 2.4 mg, respectively. The total dry weight (25 mg) of the fractions represented one-fourth of the original material. Fractions L_1 and L_5 were anticomplementary (AC) in the complement fixation test; fractions L_2, L_3 and L_4 were nonreactive with both schistosome antisera (early

* The initial saline soluble extract produces antibodies in experimental animals; the remaining fractions were not tested. Therefore, the term antigen is used to designate fractions that fixed guinea pig complement in the presence of sera from infected animals.

† Durrum electrophoresis cell, Beckman Instruments, Inc., Spinco Division, Palo Alto, California.

‡ The buffer consisted of 29.34 g veronal sodium, 19.42 g sodium acetate trihydrate and 180 ml of N/10 HCl in 3,000 ml of distilled water.

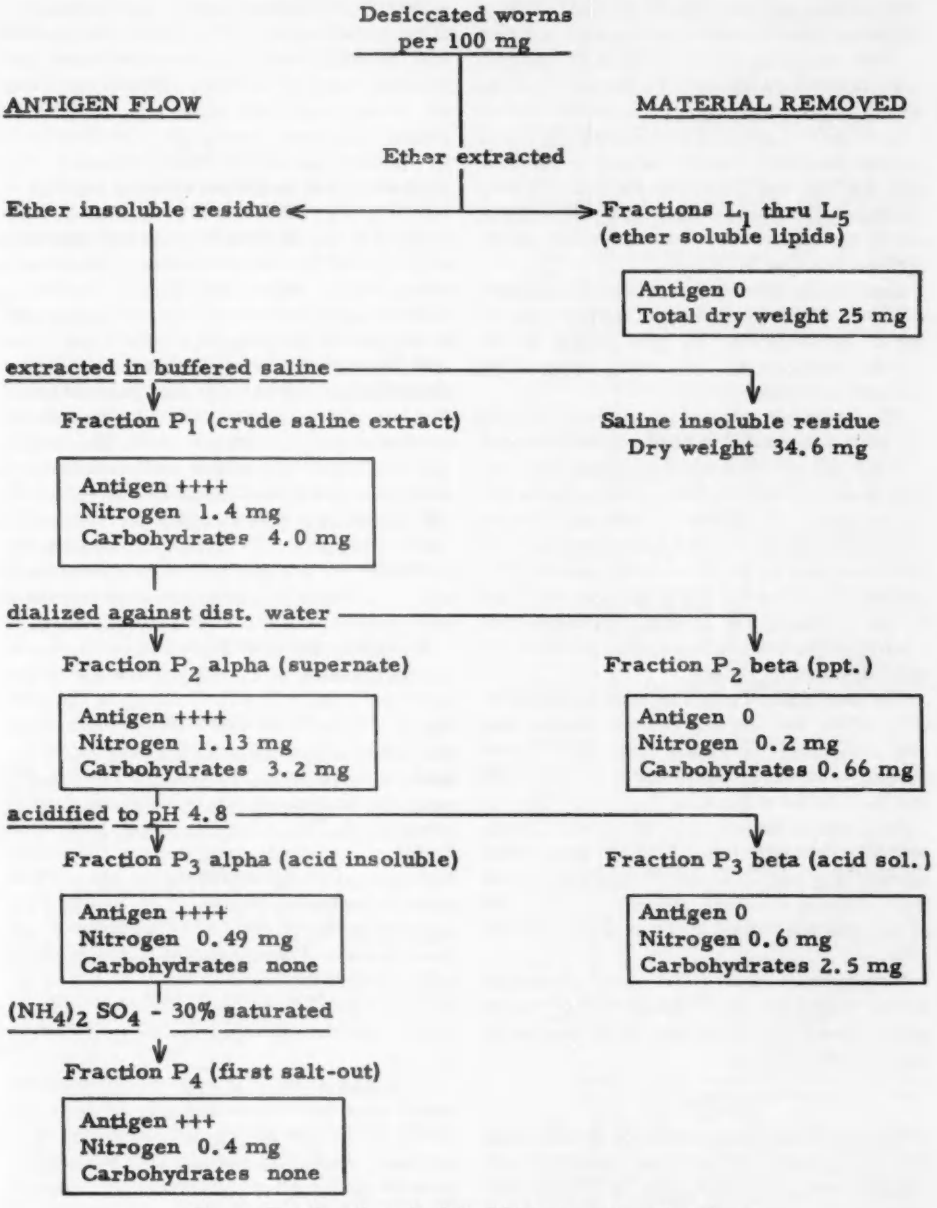

FIG. 1. Fractionation of adult *Schistosoma mansoni* antigen

infection and immune), but L_2 and L_3 were reactive with syphilitic antisera.

Protein fractions. Processing of the saline soluble crude antigen yielded seven fractions. The subscripts alpha and beta were applied to fractions resulting from a single chemical step, and represent, respectively, the antigenic and nonantigenic product of the step. The method of

fractionation and the results obtained in serological and chemical tests are presented in Figure 1. BBS extraction of the dried ether insoluble material yielded a complex (P_1) containing strong complement fixing antigen); the results obtained were considered maximal for the particular lot of antigen and were designated as ++++. Nitrogen (1.4 mg) and carbohydrates (4.0 mg) were present in the fraction. Electrophoretic analysis of the material showed 2 protein and 3 carbohydrate bands to be present.

Fraction P_2 alpha showed comparable serologic activity (++++), 1.13 mg of nitrogen and 3.2 mg of carbohydrates. P_2 beta yielded no detectable antigenicity but small amounts of nitrogen and carbohydrates.

The precipitate obtained by acidification to pH 4.8 when resuspended in BBS retained the antigenicity and was designated P_3 alpha. The fraction contained no detectable carbohydrates and the nitrogen was reduced to 0.49 mg. Electrophoretic studies of P_3 alpha showed a single protein band and no bands of carbohydrates. The carbohydrates and 0.6 mg of nitrogen remained in the non-antigenic fraction (P_3 beta); the electrophoretic fraction was similar to that of P_1, the original saline extract.

The precipitate, P_4, formed at 30% saturation of P_3 alpha, was the only salt-out fraction that was antigenic (+++) and even this fraction showed considerably less antigenic activity than did P_1, P_2 alpha or P_3 alpha.

Sera from 50 individuals with proven *S. mansoni* infections were tested with the crude saline extract (P_1) and P_3 alpha. Comparable results were obtained with both antigens; sera from all of the patients reacted to some degree in both tests.

Carbohydrates. Each 100 mg of desiccated worms yielded 5.2 mg of soluble carbohydrates which failed to fix complement in the presence of schistosomal antisera.

DISCUSSION

The ubiquitous occurrence in nature of lipoidal-like substances, which react non-specifically with syphilitic sera, was emphasized by Weil.[15] Fortunately, the non-specific lipoidal substances exhibit some degree of solubility in organic solvents. Chaffee *et al.*[5] prepared an improved antigen for a complement fixation test by briefly treating desiccated adult schistosomes with anhydrous ether prior to final extraction of the specific antigen in saline. The procedure reduced the tendency of saline soluble schistosome antigen to cross-react with syphilitic sera. The brief ether extraction, however, may not have been sufficient to remove all of the non-specific lipids.[5] In the current studies, non-specific antigenic substances were still present (L_3) after prolonged extraction with large volumes of anhydrous ether. In addition to removing more effectively the worm components responsible for the cross-reaction with syphilitic sera, exhaustive ether extraction removed anticomplementary substances. Wright *et al.*[16] attempted to employ saline extracts of schistosomes as antigen for complement fixation tests. However, the extracts were rejected because of strong anticomplementary activity. Chaffee *et al.*[5] found that brief ether extraction rendered saline extracts of schistosomes satisfactory for tests. However, in our experience, ether soluble anticomplementary substances were present in fractions L_1 and L_5. As the intermediate lipid fractions L_2, L_3 and L_4 contained no anticomplementary substances, the possibility of two distinct anticomplementary substances exists. It appears that ether extraction is required to remove both of these substances.

Apparently the most important single step in the fractionation of P_1 was acidification to the isoelectric point (pH 4.8). Contrary to the findings of Melcher[10] with *Trichinella spiralis* antigen and of Sadun *et al.*[17-19] with *S. japonicum*, *Paragonimus westermani* and *Clonorchis sinensis*, the antigenic fraction of the schistosome extract precipitated. This affords a simple method of freeing the serologic antigen from the carbohydrates and a large amount of the non-reactive material containing nitrogen, both of which remained soluble at pH 4.8. Comparison of the electrophoretic determination of P_1 and P_3 alpha indicates that the 2 protein bands and 3 carbohydrate bands in P_1 were reduced to a single protein band in P_3 alpha by the acidification process.

Serological studies of immune monkeys, not reported here, had shown that the antibody detected by routine serological tests for schistosomiasis (complement fixation, slide flocculation, cercarial agglutination and circumoval precipitation) is related to infection with the disease and titers remain apparently unchanged, later, when the animal becomes completely resistant to challenge. At the start of these studies, it was hoped that by titrating each resulting fraction against both an acute serum and an immune one that a

fraction related to immunity might be detected. However, no such fraction was found. In all instances where antigenicity occurred, the reaction was observed with both the acute and the immune sera.

SUMMARY

Prolonged ether extraction of adult schistosomes removed 25 mg of non-specific ether soluble substances from each 100 mg of worms. The lipids were collected as five fractions, of which two fixed complement with syphilitic sera and two were anticomplementary. None of the lipid fractions fixed complement in the presence of schistosome antibody. A carbohydrate fraction isolated from *Schistosoma mansoni* worms was non-reactive in the complement fixation test.

Essentially all of the complement fixing activity of the saline extract from ether-treated adult schistosomes is present in the acid insoluble protein fraction. This fraction consists of one demonstrable electrophoretic component, and contains only one-third of the nitrogen and none of the carbohydrate present in the crude extract. The protein precipitates at 30% saturation with ammonium sulfate, but loss of some antigenicity results from this step.

ACKNOWLEDGMENTS

The authors wish to extend their appreciation to Lt. Col. William H. Meroney, MC, USA, and Commander Leo A. Jachowski, MSC, USN, for their advice and criticism of this work.

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SPLENIC REACTIONS IN SWISS ALBINO MICE TO SINGLE AND MULTIPLE INFECTIONS WITH *SCHISTOSOMA MANSONI**

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The role played by the spleen in schistosomiasis has been the object of several investigations in both man and experimental animals. Based upon clinical and autopsy studies, some authors have concluded that the observed splenomegaly resulted from portal hypertension due to hepatic cirrhosis.[1-9] Others believe that the splenic lesions are a result of "toxins" elaborated by the parasite and its eggs.[10-14] Monasterio[15] was the first to claim that schistosomiasis was a disease of the reticulo-endothelial system.

Recently, Diaz-Rivera *et al.*[16] and Lobo[17] suggested that the splenic lesions could be a result of a hypersensitive state induced by antigenic substances produced by the schistosome and its eggs. Earlier, in 1954, Gelin and Couniot[18] stated that the splenomegaly in Banti's syndrome could be explained on the basis of immune reactions since the spleen is a producer of reticulo-endothelial cells.

Experimentally, Fairley,[19] Faust and Meleney,[20] Le Roux,[21] Brumpt and Chevalier[22] and DeWitt and Warren[23] noticed an increase in the volume of the spleen of infected animals. However, they did not agree on the interpretation of their findings. Brumpt and Chevalier, for instance, in mice experimentally infected with *Schistosoma mansoni* and *S. haematobium*, described splenic lesions produced by the schistosome eggs. They called them "irritative-toxic" lesions, and concluded that such lesions caused hypertrophy of the organ. More recently, DeWitt and Warren described in albino mice, experimentally infected with *S. mansoni*, a congestive splenomegaly syndrome which is much like that caused by portal hypertension in man.

Several papers have referred to a relative resistance in animals subjected to multiple infections with schistosomes.[24-27] A natural resistance also was described in some laboratory animals.[28,29] Inflammatory reactions in the skin have been observed in animals reinfected with schistosomes.[20,26,30-32] In the same way, a well characterized inflammatory response has been described in the lungs of reinfected animals.[33,34]

Considering the observations mentioned above, the finding of Onsy[10] that an individual subjected to multiple infections with schistosomes is under a continuous and intensive splenic reaction, and the fact that the spleen is the most important site of R.E.S., it was decided to study the splenic reactions developed in mice subjected to single and multiple infections with *S. mansoni*.

MATERIALS AND METHODS

One hundred and ten Swiss albino mice, about 2 months old, obtained from the Centro de Pesquisas Aggeu Magalhães colony, were used. They were infected with cercariae from *Australorbis glabratus* naturally infected with *S. mansoni*, collected in the neighborhood of Paulista, State of Pernambuco (Brazil). All the cercariae used were identified as *S. mansoni*.

The mice were infected by the Olivier-Stirewalt technique.[35] Each animal received approximately 150 cercariae. Four groups of animals were used as follows: group I, 10 non-infected animals (controls); group II, 70 animals with a single infection; group III, 20 weekly reinfected animals; and group IV, 10 daily reinfected animals.

The animals in group II were autopsied from the 3rd to the 79th day after infection to observe the early and later lesions; the spleens were weighed as shown in Table 1, and studied histologically.

Mice in group III were subjected to weekly reinfections from the 60th day of infection on, with the same technique and the same number of cercariae. Twenty-four hours after each reinfection three animals were sacrificed and their spleens were weighed as shown in Table 2.

The animals from group IV were reinfected after 60 days on 5 consecutive days with the same number of cercariae and by the same technique of infection. All animals were sacrificed 24 hours after the last infection; the weights of the spleen are recorded in Table 3.

* This investigation was partially supported by a research grant from the National Research Council of Brazil.

All the spleens were fixed in Bouin's fluid and for the histological study the following staining techniques were applied: hematoxylin-eosin, Maximow's hematoxylin-azur II-eosin, Mallory-Russell's trichrome, and silver impregnation of the reticular fibers according to Perdrau's method.

The livers of all mice were examined histologically to determine the degree of infection.

Some of the originally infected animals were discarded because of autolysis.

RESULTS

Weight of the Spleen

Group II (single infections of 150 cercariae). Table 1 shows the average spleen weight of animals that were sacrificed or died in 10-day periods.

Analysis of the results indicates no significant differences among the animals from the same group; the variation of the spleen-weight within each group is greater than that of the average weight. However, there is a significant difference between the average spleen weight of the mice of group I and that of the mice from group II.

Group III. Table 2 shows the average spleen weight of mice exposed to cercariae one to five times at weekly intervals and sacrificed in groups of three, 24 hours after each reinfection. Comparison of the averages shows no significant differences. The variation within the groups is greater than that in the average. However, the difference between the average of this group and that of normal non-infected mice in group I is significant.

Group IV. All the animals from this group were sacrificed 24 hours after the last reinfection, except two animals that were discarded because they had been found dead and in process of autolysis. The difference between the average spleen weight of this group (Table 3) and that of group I is significant; but there is no significant difference between the average spleen weight of group II and that of group III; *i.e.*, the animals subjected to weekly reinfections had an average spleen weight similar to that of the animals with a single infection. On the other hand, the average spleen weight of the animals which had been

TABLE 1

*Average weight of the spleen of mice subjected to a single infection (group II)**

Exp. group	Mice		Spleen
	No.	Days infected	Mg avg. wt. ± S.D.†
1	7	0–9	203 ± 115
2	10	10–19	197 ± 75
3	8	20–29	180 ± 80
4	7	30–39	220 ± 79
5	8	40–49	292 ± 84
6	8	50–59	178 ± 59
7	0	60–69	—
8	7	70–79	205 ± 57
Total.....	55		211 ± 22

* Average weight of the spleen of non-infected mice (group I) was 66 ± 11.1 mg.

† Standard deviation.

TABLE 2

Average weight of the spleen of mice reinfected weekly (group III)

Exp. group	Mice		Spleen
	No.	Times reinfected	Mg avg. wt. ± S.D.*
1	3	1	221 ± 64
2	3	2	278 ± 144
3	3	3	216 ± 75
4	3	4	166 ± 34
5	3	5	282 ± 15
Total.....	15	1–5	233 ± 45

* Standard deviation.

TABLE 3

Weight of the spleen of mice reinfected daily for 5 consecutive days (group IV)

Mouse no.	Spleen wt. in mg
1	409
2	400
3	323
4	414
5	349
6	400
7	350
8	450
Mean.....	387
Standard deviation.....	±39

subjected to daily reinfections (group IV) is significantly greater than that of group II or group III.

Histological Structure

Group I (*non-infected control animals, Figures 1-3*). The spleen is surrounded by a fibrous capsule with some smooth muscle cells. From the capsule, thin trabeculae penetrate into the organ, making with the reticular fibers a network whose spaces are filled with the splenic pulp.

The splenic artery by successive divisions leads to the capillaries which supply blood to the lymphatic tissue (white pulp). This surrounds the arteries like a continuous sheet, forming thickenings around the points where new vascular branches arise.[36] These nodules of lymphatic tissue with a central arteriole are called "Malpighian corpuscles." These, depending upon their state of activity, have a lighter central area where young lymphocytes, usually undergoing mitosis, can be seen. This central area is surrounded by a denser ring of small mature lymphocytes. The spaces surrounding the terminal venous sinuses are filled by the red pulp forming the splenic cords.

Reticular fibers, primitive reticular cells and fixed macrophages form a supporting framework. Besides the lymphatic elements and circulating blood cells, free macrophages, small groups of myelocytes, erythroblasts and plasma cells are also present. Since the red pulp of normal mice contains many lymphocytes and few erythrocytes, it is not well demarcated from the white pulp. Megakaryocytes are commonly found, although their number varies considerably from animal to animal.

Group II. From the 3rd day of infection on, one could observe a slight hyperplasia of the lymphatic follicles, which appeared to be more active (Figures 4-6). Frequently, mitosis occurred around the edges of the nodules where large lymphocytes predominated. The red pulp contained a large number of lymphocytes, plasma cells, polymorphonuclear cells and megakaryocytes. All these cells, normally found in the mouse spleen, were increased in number. The reticular framework seemed to be slightly distended with these cells free within an edematous red pulp. There was also an increased number of red blood cells (slight congestion) and a fibrin-like amorphous substance precipitated in fine granules. A dark brown pigment free or phagocytosed by macrophages was also found. In those mice sacrificed in the later stages, intensification of these changes was evident.

In the animals sacrificed 15 days after exposure, lymphocytic hyperplasia was apparent as a great excess of dense lymphatic tissue. Mitosis, especially among the large and medium size lymphocytes, was very prevalent and the larger lymphocytes became increasingly numerous in the nodules, while the smaller ones formed a thin layer at the periphery. The reticular framework was filled by an increased number of lymphocytes, especially of the medium and larger sizes. The sinuses were dilated and filled by a small number of polymorphonuclear and plasma cells, and a large number of megakaryocytes and lymphocytes.

There was also an increased number of fixed reticular cells constituting the sinus endothelium. These cells displayed a clear, vesicular nucleus, a prominent nucleolus and scanty cytoplasm. Frequently they were square and arranged in lines, but they also could be found free in the sinuses. Most of them were in mitosis. The congestion, when present, was slight and the trabeculae and the capsule of the organ did not seem to be thickened. Sometimes, fine granules of a dark brown pigment could be seen in large amount, free or phagocytosed by macrophages.

In mice sacrificed 25 days after infection it was evident that, besides the above described changes, there was increased congestion. The venules were dilated and engorged with red blood cells, as was the red pulp.

At 38 days after exposure an egg of *S. mansoni* was first seen in the spleen. The same described reaction was present, *i. e.*, there was an increased number of reticular and lymphatic cells; the red pulp was congested and contained a large number of polymorphonuclear cells. There was no granulomatous reaction around the egg, which showed a shrunken shell; but there was a clear area which separated the egg from the surrounding tissue. In mice which survived longer eggs were present in the spleen. Some contained a degenerating miracidium partially phagocytosed by macrophages without granulomatous formation, while others were surrounded by the typicla granuloma. The hyperplasia of the reticular cells seemed to be more intense when there was a granulomatous formation around the egg. Con-

versely, the number of polymorphonuclear cells tended to decrease.

Eggs were found in only 10 of 70 group II mice. In two cases the eggs were surrounded by a granulomatous reaction, similar to that seen in other organs. In both cases, the eggs were represented by a shrunken shell invaded by lymphocytes and polymorphonuclear cells. Usually the eggs were isolated; in only one case were they grouped. They were always near the capsule, generally in the red pulp, and never inside the follicles.

Group III (*mice reinfected weekly*). The microscopic examination of these spleens showed changes similar to those in the last group. However, the lymphatic nodules generally were more hyperplastic and were poorly delimited from the surrounding tissues. The lymphatic cells were all in a young stage, many exhibiting mitosis, and small lymphocytes could seldom be seen.

The red pulp, as in the spleens of the animals from group II, was intensively cellular and slightly congested. The reticular cells were markedly increased in number and size, and many of them were undergoing mitosis. Megakaryocytes were also more numerous, but polymorphonuclear leukocytes were rare. No eggs were found in spleen sections from any of the 15 mice in this group. The splenic capsule and trabeculae frequently were swollen and the capsule sometimes contained thickened areas of fibrosis.

Group IV (*mice reinfected daily*). The spleens of the mice from this group weighed more than those of mice in the other groups. Histological examination revealed that the increase in volume was associated with marked hyperplasia of the lymphatic and reticular elements (Figures 7–9). Thus the splenic capsule, the trabeculae, and the reticular framework were swollen and sometimes thickened by fibrous tissue.

The lymphatic nodules were poorly differentiated from the red pulp, since they were formed only by large lymphocytes. These cells were abundant and were mixed with the reticular cells, monocytes and other elements of the red pulp. It was difficult to make a precise distinction among these different elements. However, the lymphocytes seemed to be less numerous than the reticular cells. These cells lay along the endothelium or were free, and many of them showed mitosis. A large number of monocytes and megakaryocytes were also present in the red pulp. Plasma cells and polymorphonuclear leukocytes also were present but scanty; sometimes eosinophils predominated. Because of the dense cellularity of the tissues, the red blood cells could hardly be found in the red pulp. Pollak's trichrome staining revealed some fibrous thickening of the trabeculae and reticular framework.

DISCUSSION

The results obtained with the mice given single infections demonstrated that from the 3rd day on after exposure the spleen changes were characterized by a slight lymphatic hyperplasia.

Faust, Jones and Hoffman,[37] who studied the picture of schistosomiasis in rats, noted that it was exactly on the 3rd day that the first schistosomulae started to leave the lungs, entered the general circulation, and thus initiated the toxemic stage which lasted about a fortnight. During the migration of the schistosomulae in the body of the host many apparently are lost in the supporting connective tissue before reaching the portal vessels (about 80% in mice[38,39]), but this is not an evidence of resistance. There is, however, evidence of an increase in the mortality rate of parasites in the early stages of the infection in resistant animals.[40]

The splenic reactions observed in the present experiments became progressively more intense and reached a high degree around the 15th day, at which time the lymphatic and reticular hyperplastic reaction was very prominent. There seems to be no doubt that, at this stage, a splenic reaction took place against the invading larvae, in "septicemic" form, being probably part of a toxic or general irritative state. In fact, a similar reaction was described by Brumpt and Chevalier[22] in the spleens of mice infected with *S. mansoni* and *S. haematobium* during the late prepatent stages of the infection.

In regard to the phagocytic function against foreign substances which invade the blood, the spleen is very active, because of the action of the macrophages, as can be seen, for instance, in malaria. When the infection lasts for a longer period, the hyperplasia of the reticular cells seems to be intensified, probably as a result of greater phagocytic activity against the action

of the adult parasites and their eggs on the tissues of the host.

No definite resistance was observed in animals against a second infection until the first one became mature. This points to the advantage of destruction of eggs or parasites within the tissues, with the resulting liberation of antigenic substances in a sufficient amount to stimulate specific antibodies. This would seem to explain the greater hyperplastic reaction observed in the spleens of the mice which were sacrificed in the later stages of infection.

In the group of animals subjected to multiple reinfections, an intensive lymphatic and reticular hyperplasia was demonstrated, characterized by the presence of young cells and great mitotic activity. This quantitative increase in endothelial fixed cells of the venous and lymphatic sinusoids, of other reticular cells and of free macrophages showing proliferative activity indicates a true "mesenchymal activation," which is frequently associated with splenomegaly.

It has been demonstrated that in experimental animals a resistance against the schistosomes occurs after one or several reinfections.[24-27,32,33,41] Although there is not an absolute resistance, a limitation of the number of parasites takes place through the destruction which they suffer in the skin and lungs, only a portion being allowed to reach the adult stage.

Comparison of the reactional aspects of the spleens of mice in the group given single infections with those of the two reinfected groups revealed that there was greater hyperplasia in the reinfected groups, and that the hyperplasia became considerably increased in the animals subjected to reinfections every 24 hours.

The animals from this last group were almost continuously exposed, resulting in a state of permanent reaction of the splenic reticulo-endothelial system. The splenomegaly of these animals (demonstrated in Table 3) confirms the reactions shown by the histological picture. Thus through the results obtained in the present experiment one can conclude that the role played by the spleen in mice experimentally infected with *S. mansoni* is the result of a reaction of the reticulo-endothelial system against foreign proteins liberated by worms and their eggs.

The presence of eggs in the interior of the splenic parenchyma as a cause of the splenomegaly has been claimed by some authors.[10,32] However, with the results obtained in this experiment, one can conclude that the splenic reactions do not depend on the presence of eggs in the tissues of the spleen. When present, the eggs could either provoke a granulomatous reaction or not; but the presence of a diffuse reaction of the spleen, even in the early stages of the infection, before the parasites started to lay their eggs, was a demonstration of the action of the spleen against circulating products originating from the parasites.

Finally, considering the splenic changes observed in the animals of the present experiment in relation to the opinions of the authors mentioned in the beginning of this paper, the following conclusions are drawn: (1) the splenic changes were characterized by a lymphatic and reticular hyperplasia; (2) this hyperplasia began in the "septicemic" (acute) stage of the disease, against the invading larvae; (3) there was an increase of the hyperplasia of the splenic R.E.S. in the later stages of the parasitosis as a result of the destruction of the parasites and their eggs; (4) the congestion observed in the spleen of the animals which died after the 25th day of infection was probably a result of the mechanical action of the living worms, causing obstructions in the intrahepatic portal circulation; (5) the marked increase of both fixed and free reticular cells seen in the spleen of the reinfected mice represented a so-called "mesenchymal activation" also described in other parasitoses;[42] (6) such a "mesenchymal activation" gave rise to an increasing phagocytic activity, thus suggesting a probable resistance acquired by the mice against new infections; and (7) the splenomegaly in experimental schistosomiasis, and probably also in man, is a result of a combination of the reticulo-endothelial hyperplasia of the spleen, added to a secondary congestion caused by portal hypertension.

SUMMARY

One hundred and ten Swiss albino mice in three groups were experimentally infected with *Schistosoma mansoni* cercariae. In the first group the spleen weight was recorded from the 3rd to the 79th day after one exposure. The animals in the second group were reinfected weekly beginning 60 days after the primary infection, and the average spleen weight was recorded. The third group was reinfected daily, beginning 60 days after initial exposure.

The average spleen weight in the three groups,

compared with that of a control group, increased (splenomegaly), markedly so in the daily reinfected animals. Histologically there was lymphatic and reticular hyperplasia which began on the 3rd day after infection (toxemic stage). In animals which survived from the 40th day on, the lesions were more evident, probably as a result of greater phagocytic activity due to the destruction of adult parasites and eggs in the tissues of the host. In animals reinfected weekly the splenic lesions were similar to those in the first group, but the hyperplasia was more intense. In the group of mice reinfected daily, the intensity of the lymphatic and reticular hyperplasia was even greater.

ACKNOWLEDGMENT

We wish to express our thanks to Dr. Jandyra de Moraes Barbosa, from the Centro de Pesquisas Aggeu Magalhães, for her help in statistical analysis of the data.

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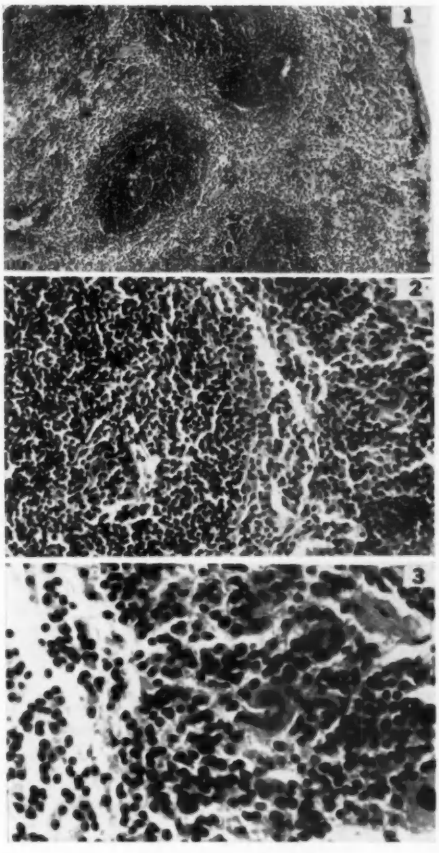

PLATE I

FIGS. 1–3. The histological pattern of the spleen in normal mice.

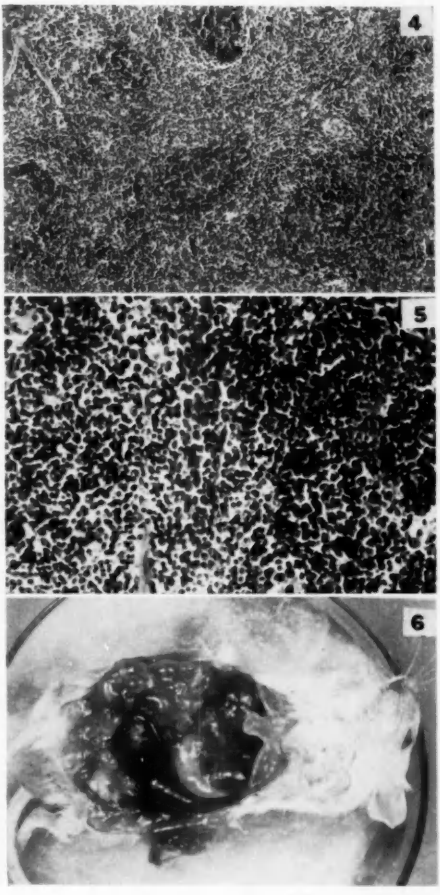

PLATE II

FIGS. 4 and 5. The splenic hyperplasia of mice with a single infection. Comparing Figure 4 with Figure 1, a slight blurring of the lymphatic follicles due to the increased cellularity of the red pulp is evident. In Figure 5 the numerous large and medium sized lymphocytes in the follicle and in the red pulp demonstrate the hyperplastic stage of the spleen.

FIG. 6. Enlarged spleen of a mouse reinfected daily.

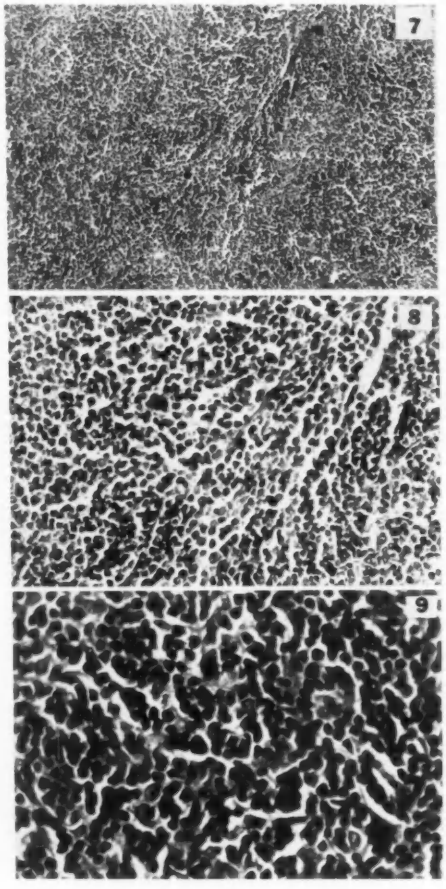

PLATE III

FIGS. 7-9. The splenic changes in reinfected animals. In Figure 7, the splenic pulp shows such a massive number of young lymphocytes that the lymphatic follicles cannot be identified. Figure 8 shows the splenic reticular framework with hyperplasia of the reticular cells. In Figure 9, the large number of young lymphocytes and reticular cells indicates the intensity of the splenic hyperplasia.

All photographs were made with a Reichert microscope: Figures 1, 4 and 7 with × 10 ocular and × 6.3 objective; Figures 2, 5 and 8 with × 10 ocular and × 30 objective; and Figures 3 and 9 with × 10 ocular and × 60 objective.

THE EFFECT OF TWSb ON THE ELECTROCARDIOGRAM OF CHILDREN SUFFERING FROM URINARY BILHARZIASIS

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It is a well established fact that the ordinary treatment of bilharziasis with tartar emetic and Fuadin® (stibophen) entails a variable strain on the heart and that the changes induced in the electrocardiogram by tartar emetic are more common and of greater degree than are those induced by stibophen.[1] Khalil[2] estimated the mortality rate to be 0.5% with tartar emetic and 0.2% with Fuadin; in 1936, the same author[3] estimated that in Egypt about 2,000 individuals a year are lost through one million courses of tartar emetic.

Consequently, newer antimony preparations that are likely to be less toxic are always welcomed. Recently a new compound, antimony-a,á-dimercaptosuccinate (TWSb), has been introduced for the treatment of schistosome infections. Like the more toxic drugs, it is a trivalent antimonial but with two attached sulfhydryl groups which exert a detoxicating action on antimony akin to that of BAL. In mice its acute toxicity was found to be one-tenth that of Fuadin and one-fiftieth that of tartar emetic.[4]

The drug was first used in the treatment of bilharzial infection by Friedheim *et al.*[4] They reported that, of 134 patients, none showed clinical disturbances of the circulatory system and none showed any significant electrocardiographic changes. They treated children with 3 doses, totaling 0.04 g/kg body weight, during a 48-hour period. Alves[5] gave to school boys, weighing 27 to 57 kg, intravenous injections of 0.4 g of TWSb daily for 4 consecutive days, *i.e.*, a total of 1.6 g TWSb irrespective of body weight, and claimed that this produced no objective signs of any kind. Salem and Friedheim[6] recommended a total dose of 40 to 60 mg/kg for children to be given during a 5- to 7-day period.

A review of the literature reveals that since the first report of Friedheim *et al.*[4] there have been no reports, confirmatory or otherwise, on the effect of the drug on the electrocardiogram.

The present work is a study of the effect of the drug on the electrocardiogram in 17 Egyptian children suffering from urinary bilharziasis.

MATERIALS AND METHODS

Seventeen children, 6 to 13 years old, with urinary bilharziasis were selected from the outpatients of Abbassia Children's Hospital. They were all subjected to a thorough clinical examination; with the exception of one who had anemia with 50% hemoglobin, none had significant diseases other than bilharziasis.

The drug was administered by the intramuscular route in 5 equal doses totaling 0.04 g/kg body weight, which at the present time is the smallest total dose recommended for children. To observe the effect of spacing in doses the patients were divided into 3 groups of 5, 4, and 8, respectively: those in group I were given the drug on 5 consecutive days; group II, on alternate days; and group III, twice weekly.

Every patient had an ECG tracing prior to treatment and this served as a control from which all subsequent changes were noted. Subsequent ECG records were taken after each injection (except on Fridays) in the first group and after each 2 or 3 injections in groups II and III. Additional tracings were taken after periods of 1 week and 4 weeks to see whether the changes in the ECG were recoverable or not. During the study 100 tracings were taken for the 17 cases.

All the tracings were taken under standard conditions, with the patient lying in a comfortable prone position. The standard gauge of amplitude was always 1 MV. = 10 mm. The records were usually taken the day following the injection and occasionally 1 to 2 hours after the injection.

All patients were cured. The details concerning the drug's action on the parasite are the subject of a separate paper.[7]

RESULTS

Analysis of the 100 ECG tracings taken before, during and after treatment with TWSb revealed that in all the cases studied the heart rate, the rhythm, the P-wave, the P-R interval and the QRS complex were practically unchanged. The changes noted were confined to changes in the

TABLE 1

The relative frequency of T-wave, S-T segment and Q-T ratio changes in groups of children given TWSb daily, on alternate days, or twice weekly

Group (and no. patients)	Drug given	Changes in			Rate of recovery after treatment
		T-wave*	S-T segment	Q-T ratio	
I (5)	Daily	Negative to V_6 in 1, to V_5 in 2, to V_4 in 2, and in L_{III} in 1; flat in L_I, L_{II}, L_{III}, AVF and AVL in 1 case	Coving of S-T in all precordial leads where T-wave became negative	Slight prolongation in 4 cases	*After 1 week:* negative T-wave persisted in V_1 and V_2 in all cases, up to V_4 in 1 case (with anemia) *After 1 month:* negative to V_4 in 1, to V_2 in 1, and to V_1 in 3 cases
II (4)	On alternate days	Negative to V_6 in 1, to V_3 in all others; flat in L_I, L_{II}, L_{III}, AVF and AVL in 1 case	Coving in 2 cases	Slight prolongation in 2 cases	*After 1 week:* T-wave negative to V_2 in all cases *After 1 month:* return to initial tracing in 2; still slightly negative to V_2 in 2 cases
III (8)	Twice weekly	Negative to V_4 in 2, to V_3 in 4, to V_2 in 2, and in L_{III} in 4; flat or negative AVF in 2; flat AVL in 1 case	Coving in 3 cases	Slight prolongation in 1 case	*After 1 week:* return to initial tracing in 1; negative to V_3 in 1, to V_2 in 3, and in V_1 in 3 cases *After 1 month:* return to initial tracing in 5; still slightly negative in V_1 in 3 cases

* Reduced amplitude in all leads that are not listed.

T-wave, S-T segment and Q-T ratio. The T-wave was the component of the ECG which showed the most striking changes during treatment. It was affected in all cases. In some, it was reduced in amplitude while in others it became sharply negative. The S-T segment showed an upward convexity (coving) in all the precordial leads in which the T-wave turned negative in group I, in 2 of the 4 cases in group II, and in 3 of the 8 cases in group III. On the other hand, the Q-T ratio showed slight prolongation (above 1.01) in 4 of the 5 patients in group I, in 2 of the 4 in group II and in 1 of the 8 in group III. Table 1 shows the relative frequency of the T-wave, S-T segment and Q-T ratio changes with the different plans of treatment adopted.

The tracings taken 1 and 4 weeks after treatment showed that the changes noted were of a reversible nature. It was found that the more widely the subsequent doses were spaced, the less frequently ECG changes were induced and the earlier the recovery (Table 1).

Since the mere description of the curves is insufficient to convey a clear picture of the ECG changes evoked by the treatment, two figures are given as examples of the pathological development of the ECG during treatment (Figures 1, 2).

DISCUSSION

From the above investigation it is evident that the administration of TWSb in the smallest doses recommended for treatment of bilharziasis in children[4–6] induces marked changes in the ECG tracings. No symptoms, however, were observed in this series of cases. The ECG changes were noted in every patient receiving the drug but were more common and more marked in those taking it daily. In this respect it is of interest that Tarr[1] in a similar study of tartar emetic and

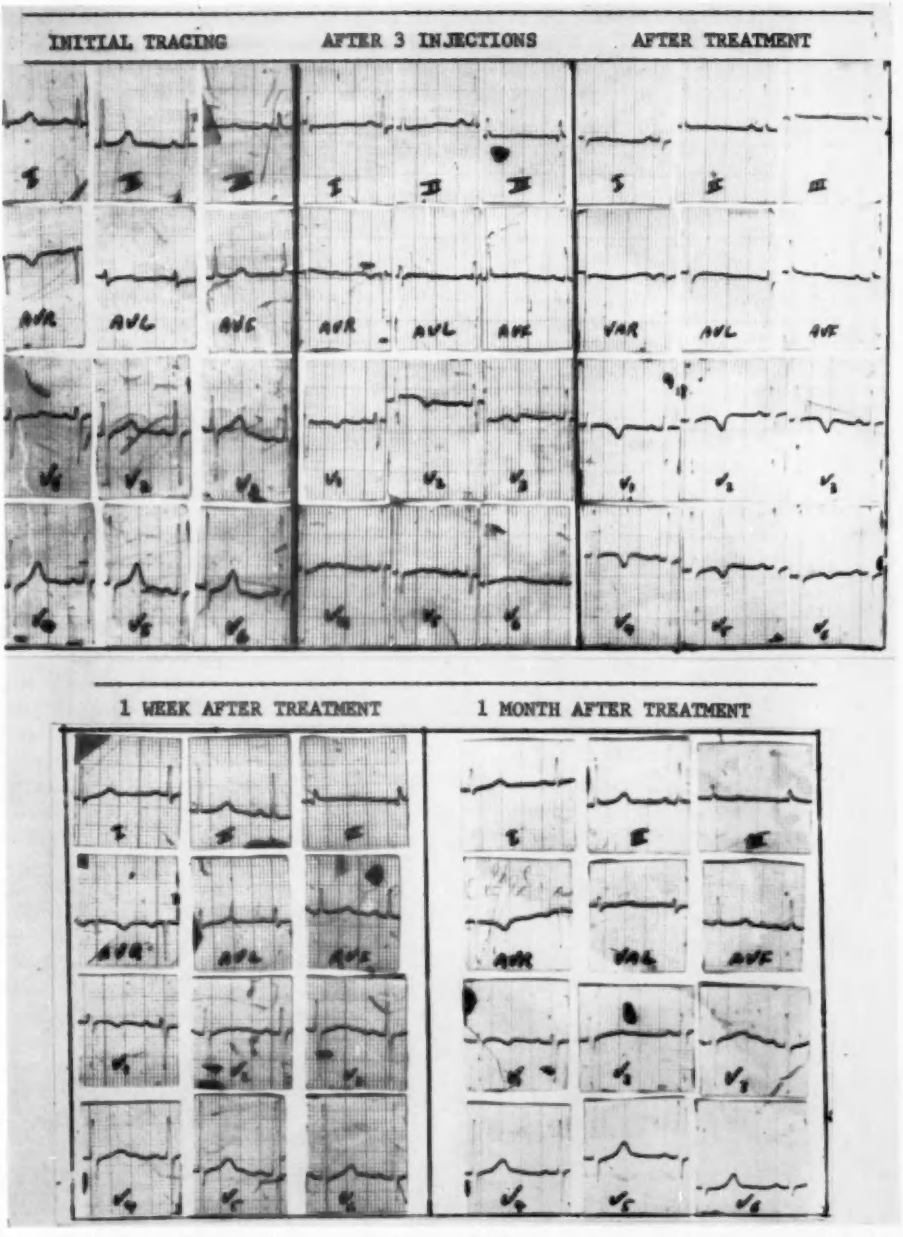

FIG. 1. Electrocardiograms from case 1 of group I before, during, and after five consecutive daily injections of TWSb.

Fuadin noticed ECG changes in every patient receiving tartar emetic and in 57 to 80% of those receiving Fuadin.

The ECG changes noted with TWSb are very similar to those described by other authors with tartar emetic and Fuadin. Mainzer and Krause,[8] who were the first to report significant ECG changes in man following the administration of

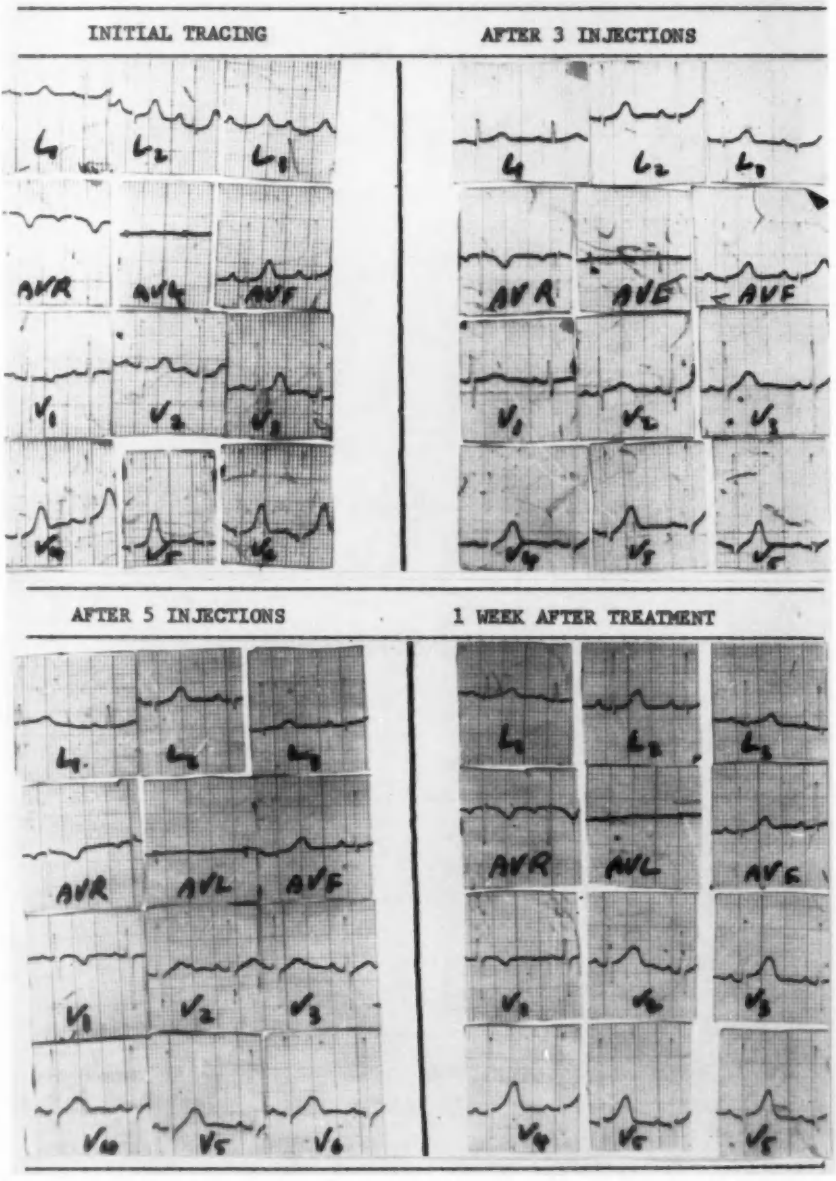

FIG. 2. Electrocardiograms from case 15 of group III before, during, and after twice weekly injections of TWSb to a total of five injections.

tartar emetic, noticed that most of the alterations occurred in the T-waves and S-T segment. Magalhães and Dias[9] in a study on the effect of antimony therapy for schistosomiasis on the ECG in adults described similar T-wave changes and found prolongation of the Q-T interval in 27% of their cases. Schroeder *et al.*[10] reported on similar T-wave changes and on fusion of the S-T segment and T-wave. In Egypt, Kenawy[11] found that the changes were mainly in the T-wave in adults while Gholmi *et al.*[12] found that the changes were confined to the T-wave and Q-T ratio in children. Tarr[1] reported on the marked similarity of the ECG changes induced by tartar emetic and by Fuadin.

In this series the changes in the T-wave were more common and more marked in leads V_1, V_2, and V_3 than in V_5 and V_6 indicating greater involvement of the right side of the heart. The same observation was made by Gholmi *et al.*[12] Anrep *et al.*[13] experimenting on dogs found that antimony preparations led to pulmonary stagnation and increased permeability of the lung capillaries, conditions which in turn increased the resistance to the right heart. However, in the present work x-rays taken before and after treatment failed to show any pulmonary changes. Apparently therefore, it is the right side of the heart which is more vulnerable to the toxic effect of the drug.

That the toxicity of the drug is not due to the potassium radicle in the molecule is evident from the fact that there is no similarity between the changes noted here and those induced by potassium, and also from the fact that the Fuadin molecule contains no potassium and produces similar ECG changes.

Finally, it was noted that the ECG changes induced by TWSb were of a reversible nature (Figures 1, 2) and that the more widely the subsequent doses were spaced, the earlier was the recovery. On the other hand anemia tended to retard recovery as noted in one case.

SUMMARY AND CONCLUSIONS

TWSb was employed in the treatment of 17 Egyptian children suffering from urinary bilharziasis. The drug was given daily, every other day or twice weekly. Observations on its effect on the electrocardiogram indicated that giving the drug is safer in twice weekly than in more frequent doses and is, moreover, equally effective.

ACKNOWLEDGMENT

The authors are indebted to Dr. H. F. Nagaty, Professor of Parasitology and Chairman, Department of Endemic Diseases, Faculty of Medicine, Ain Shams University, for kindly supplying the drug (TWSb) used in this study.

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DEMONSTRATED CONTROL OF *AUSTRALORBIS GLABRATUS* BY *MARISA CORNUARIETIS* UNDER FIELD CONDITIONS IN PUERTO RICO*

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Eradication or control of *Australorbis glabratus*, the intermediate snail host of *Schistosoma mansoni*, by *Marisa cornuarietis* has been suggested by recent investigations.[1–5] These studies showed that the voracious feeding habits and rapid multiplication of *Marisa* depleted the food supply available to *Australorbis* resulting in a drastic reduction. From laboratory evidence[2] it is assumed that *Australorbis* egg masses and small snails are consumed by *Marisa* with the aquatic vegetation.

The purposes of the present study were to determine quantitatively the effect of *Marisa* as a biological control agent on a thriving *Australorbis* population in ponds with heavy aquatic vegetation and to study the interrelationship of the two snail populations.

MATERIALS AND METHODS

A suitable study area was found on the property of the U. S. Department of Agriculture, Agricultural Experimental Station, Mayagüez, Puerto Rico (Figure 1). The field plot was a small natural watershed that had harbored a colony of *Australorbis* snails for many years. The watershed had a chain of five ponds, each with a dense foliage consisting primarily of ornamental lilies (*Nymphaea ampla*) estimated to weigh 2 to 5 tons per pond. Pond E, containing 1 to 2 million gallons of water, was the largest and served as a reservoir for the remaining ponds, successively below it, which contained 125,000 to 200,000 gallons of water each. Frequent heavy rains caused flooding of the ponds but with little apparent influence upon the *Australorbis* population which was well protected by the heavy vegetation. Parasitized *Australorbis* were found in ponds A and B where *S. mansoni* infection rates up to 25% prevailed at the start of the study.

Ten collecting sites, each 50 feet long, were marked at selected places along the margins of the ponds (Figure 1). Preliminary snail collections were made at these sites during September, 1957, to determine the density of the *Australorbis* population before the introduction of *Marisa*. Collections were repeated in 3 weeks (October) and then 200 medium to large laboratory pond reared *Marisa* were released into each pond; no *Marisa* were planted in the connecting channels.

Snail collections at each designated station were repeated at 6-week intervals. Each collection site was sampled by making 25 dips with a standard wire mesh scoop measuring one-half square foot. All *Australorbis* and *Marisa* collected were counted, measured, and, with the exception of infected *Australorbis*, returned to the sites where collected. For convenience, the *Marisa* were classified into three groups: small (3 to 10 mm), medium (11 to 30 mm), and large (31 to 60 mm).

Observations on the density of the two snail populations and the vegetative growth continued for 81 weeks.

RESULTS

Marisa continued to propagate, producing egg masses in abundance throughout the entire period. After the first two collections the numbers of these snails steadily increased until the 63rd week after which no further increase was noted. Approximately 60% of the *Marisa* collected throughout the observation period were "small" indicating propagative activity.

The *Australorbis* population reached peaks of reproduction at approximately the 9th and 45th

* A preliminary report was presented before the 8th annual meeting of the American Society of Tropical Medicine and Hygiene, 29 October 1959, Indianapolis, Indiana.

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FIG. 1. Schematic diagram of the area under observation at the Federal Experimental Station, Mayagüez, Puerto Rico.

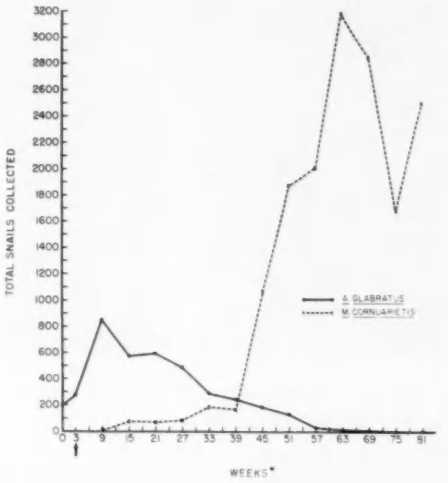

FIG. 3. Population changes of *Australorbis glabratus* in a series of joined ponds after introduction of *Marisa cornuarietis*. (↑), *M. cornuarietis* were introduced. (*), each 6-week collection represented the total number of snails recovered from 250 dips with the wire mesh scoop.

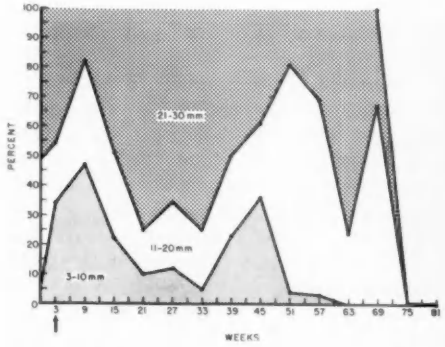

FIG. 2. The relative proportion of various sizes of *Australorbis glabratus* following the introduction of *Marisa cornuarietis*.

FIG. 4. Pond C, harboring a dense growth of ornamental lilies typical of the other ponds.

weeks (Figure 2), made evident by the increased proportion of small snails. However, despite these propagative periods the density of *Australorbis* declined steadily while the *Marisa* population increased rapidly (Figure 3). The numbers of *Australorbis* from ponds A and B that were shedding *S. mansoni* cercariae also decreased until the 57th week after which no positive snails were collected. No living *Australorbis* was found after the 75th week.

The influence of the *Marisa* upon the foliage in the ponds was striking. In pond C the heavy growth of lilies (Figure 4) was essentially eradicated in 51 weeks (Figure 5). Pond D was cleared by the 63rd week and all other ponds were free of foliage by the 75th week. Connecting channels

FIG. 5. Pond C, freed of ornamental lilies within 51 weeks by *Marisa cornuarietis*.

FIG. 6. Accumulation of *Marisa cornuarietis* characteristic for all channels.

were rock-riveted and offered little food to *Marisa* although many were washed into them during storms (Figure 6).

DISCUSSION

The introduction of a relatively small number of *Marisa* into an area of heavy vegetation with a well established *Australorbis* population was considered a critical test of the former as a biological control agent. The time required for the *Marisa* to destroy the vegetation and the *Australorbis* population was approximately 1.5 years; these results are considered to be dramatic. During the first 39 weeks following the introduction of the *Marisa*, the slow but steady increase in population of these snails resembled the lag phase in the biological cycle; after this time the population increase was apparently in the logarithmic phase (Figure 3). This latter phase occurred simultaneously with a propagative peak in the *Australorbis* population as determined by the increased numbers of small *Australorbis* snails recovered (Figure 2). Concurrently, the destructive influence of the *Marisa* upon the foliage was becoming increasingly evident. As no further population increases in the *Australorbis* were observed, it appears that the *Marisa* devoured the *Australorbis* eggs and young snails while feeding on the foliage. After 63 weeks no small snails were observed at any of the collection sites.

Any direct effect of the *Marisa* upon the adult *Australorbis* could not readily be determined. However, at 51 weeks *Marisa* were found feeding on the shells of living *Australorbis* as evidenced by scarification of the latter. This phenomenon was not observed at any other time during the study.

Subsequent to the quantitative phase of the investigation an intensive survey was made of the entire watershed and it was found that a few *Australorbis* still existed in the channels joining the study ponds, in a few small paralotic marshes, and in a previously unsurveyed headwater lake. This status prevails to date (September, 1960) and gross observations are being continued indefinitely on a quarterly schedule. The persistence of *Australorbis* in the study area may be due to seeding from lateral and headwater sources since there has been no visual evidence of propagative activity within the area.

SUMMARY

A relatively small number of *Marisa cornuarietis* was introduced into a chain of five ponds containing well established *Australorbis glabratus* populations. During an 81-week observation period the *Marisa* effectively reduced the *Australorbis* population almost to extinction. The population has not recovered in one and a half years. Additional studies are indicated to

determine if *Marisa* will work equally well in other *Australorbis* habitats.

ACKNOWLEDGMENTS

The authors are indebted to Lt. Col. William H. Meroney, MC, USA, for suggestions and criticisms. We wish to acknowledge the technical assistance of the following persons in this study: D. G. Erickson, C. S. Richards, R. S. Clark, A. Sullivan, B. A. Dent, F. Robbins, R. J. O'Brian and E. Rivera.

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EPIDEMIOLOGY OF ECHINOCOCCOSIS IN THE MIDDLE EAST

I. Human Infection in Lebanon, 1949 to 1959*

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The Middle East is frequently cited as among those areas of the world in which hydatid disease is most prevalent although published evidence for this is limited. A total of 385 human cases was reported from Lebanon, Syria, Palestine and Iraq during the 25-year period 1923 to 1949.[1]

Presented here are the results of a study on human infection with *Echinococcus granulosus* in Lebanon during the subsequent 11-year period.

Population

Politically Lebanon is divided into the 5 governorates (*mohafaza*) of North Lebanon, Mount Lebanon, Beirut, South Lebanon and Beqa'. Each of these, with the exception of Beirut, is divided into districts (*aqdiyat*). The boundaries observed here for the several districts were those indicated on the 1:200,000 map of Lebanon published in 1948 for the Société d'Encouragement au Tourisme by l'Institut Geographique National, Paris; those for the quarters of Beirut on the map of the city included in the survey by Churchill.[2]

The composition of the population is varied and reflects very much the checkered history of the country. Reliable population statistics for Lebanon are not available.

The last official census was taken in 1932 during the French mandatory period. The population was enumerated by place of registration of birth rather than by place of actual residence (Table 1). Subsequent official estimates (*Registre de l'Etat Civil*) have been based upon this 1932 census with periodic revision from certificates of births and deaths as recorded by local mayors (*mukhtars*). Revised figures to 1959 are given in Table 1. A tabulation of the records of births and deaths by year for 1944 to 1957 was published by Gholl[4] in 1958 and some of the sources of error in the official registers were discussed by that author.

In 1942, the Office des Céréales Painifiables (O.C.P.) undertook a national census for the purpose of wartime food distribution. This census was based upon actual residence and enumerated all residents of Lebanon irrespective of nationality. A comparison of population estimates for 1942 and 1944 based upon these two sources is given in Table 1. These show that official registration statistics for Lebanon tend to understate the actual resident population of the Beirut governorate and to overstate that of the remainder of the country. The over-all discrepancy for 1944 of 62,412 persons between the registration and the residence tabulations possibly reflects a portion of the sizable number of immigrants officially registered as Lebanese. Since the O.C.P. census also included some 39,681 foreigners resident in Lebanon (revision as of 1944[7]), the actual difference between Lebanese registered in and Lebanese resident in the country for 1944 would be more nearly 100,000 or approximately 10% of the registered population. There has been no more recent census comparable to that of the O.C.P.

A large number of refugees were added to the population as a result of the Palestine war of 1948. In 1957, their number in Lebanon totaled 102,586.[8]

An additional estimate of the population of the Beirut governorate was provided in a socio-economic survey of the city undertaken in 1952 and 1953.[2] Inflation of figures based upon household sampling gave a total population for Beirut of 296,029. Official registration figures for 1953 were 219,779. The ratio of the estimated resident population to the registered population for Beirut in 1953 was 1.35; this same ratio calculated for 1944 (Table 1) was 1.36. The registered population of Beirut for 1959, therefore, was corrected by a factor of 1.35 in the calculation of age specific incidence rates.

* Portion of a project on the epidemiology of zoonotic diseases in the Middle East. This study was supported in part by grants from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service (E-2205), and from Pfizer International.

TABLE 1

Estimates of the Lebanese population by governorate

	1932*	1942†	1944‡		1944§	1959‖
			Lebanese nationals	All residents		
North Lebanon	233,120	221,886	217,432	226,659	246,964	358,350
Mount Lebanon	184,007	253,931	269,540	271,713	335,774	473,148
Beirut	160,759	233,667	213,433	237,348	174,001	259,720
South Lebanon	154,752	169,207	168,418	170,237	212,848	320,026
Beqa'	122,055	143,423	155,682	158,229	157,014	238,909
Total	854,693	1,022,114	1,024,505	1,064,186	1,126,601	1,650,153

* 1932 official census of persons of Lebanese nationality by place of registration of birth.[3]

† 1942 Office des Céréales Painifiables census of all residents of Lebanon by place of residence. For comparison, the estimate for 1942, based upon the 1932 official census of persons of Lebanese nationality by place of registration, revised by local registration of births and deaths, is 1,086,824.[3]

‡ 1942 Office des Céréales Painifiables census of all residents of Lebanon, by place of residence, revised to the end of 1944.[4]

§ 1932 official census of persons of Lebanese nationality by place of registration, revised to December 31, 1944, by local registrations of births and deaths.[5]

‖ 1932 official census of persons of Lebanese nationality by place of registration, revised to 1959 by local registrations of births and deaths.[6]

TABLE 2

Official estimate of the Lebanese population by religious community (1953)[10]

	Christian	Moslem	Druze	Jew	Other	Total
North Lebanon	182,032	120,240	19	40	6,064	308,395
Mount Lebanon	302,943	47,139	71,569	95	447	422,193
Beirut	118,567	93,178	2,457	5,382	215	219,799
South Lebanon	78,225	178,334	6,893	1,108	206	264,766
Beqa'	94,845	98,701	7,193	67	261	201,067
Total	776,612*	537,592†	88,131	6,692	7,193	1,416,220

* Includes 423,706 Maronite; 149,528 Greek Orthodox; 90,788 Greek Catholic; 4,446 Roman Catholic; 68,679 Armenian Orthodox; 14,582 Armenian Catholic; 13,585 Protestant; 4,817 Syrian Orthodox; 6,013 Syrian Catholic; and 466 Chaldean.

† Includes 286,237 Sunni and 251,355 Shi'a. Historically, the Druzes are a dissident Moslem sect. At the present time, some Druzes do and some do not consider themselves Moslems.

In 1955 and 1956 a census was taken of a group of 13 villages in the Beqa' valley.[9] The total resident population for this group of villages was 8,084 as compared to an official registered population of 11,360. Results of both of these studies tend to substantiate the conclusions about registration estimates stated above.

Composition of the Lebanese population by religious group, according to official registration figures for 1953, is shown in Table 2. A somewhat more detailed treatment of the population by district, according to religious community, is given by Tabbarah.[11] A common belief is that Moslems are at present at least numerically equal to Christians, but this may or may not be true.

Survey Method

According to Ministry of Health statistics,[12] there were in Lebanon, in 1957, 139 hospitals including sanatoria, with a total of 5,869 beds. An attempt by us to revise this list would reduce the total number of hospitals for 1959 to 113 (5,853

beds). Excluded from the present survey were the specialized institutions among these, such as maternities, and hospitals which did not admit patients for major surgery. The results reported here are based upon an examination of the surgical records of 54 hospitals for the period 1949 to 1959.

Where possible the following information was obtained from the hospital records for each hydatid disease patient and entered directly or punch-coded on 5 × 8 in. marginal punched cards: (1) name of patient, (2) hospital name and case number, (3) year of operation, (4) organ involved, (5) results of immunologic tests, and the (6) age, (7) sex, (8) religion, (9) occupation and (10) place of residence of the patient. Cases which represented known readmissions for hydatid disease were discarded.

Total Surgical Cases of Hydatid Disease

Nine hundred thirty-eight surgical cases of hydatid disease from Lebanese hospitals were disclosed by this survey. Of these, 467 patients were males, 416 females and for 55 the sex was not recorded. Reasonably complete records were available for 885 cases. Of these latter, 516 patients were Christians, 245 were Moslems, 29 were Druzes, while for 95 religion was not recorded. Four hundred fifty-five of the 885 cases were from three hospitals (American University Hospital, 241; Hotel Dieu of the Faculté Française de Médecine, 130; Bhannes Sanatorium, 84). For various reasons the charts of 60 patients operated on prior to 1949 (56 of these during 1939 to 1949) were present in the hospital records surveyed. Twenty-three of these were from the American University Hospital and presumably included in the 86 cases reported by Pipkin *et al.* for 1937 to 1949.[1]

Of the total of 885 reasonably complete case records, 738 were of persons giving their residence as Lebanon, 95 were Syrians, 20 were Iraqis, 11 were Palestinians, 8 were Jordanians and 13 were residents of other Arab countries. Patients giving Palestine as their home were all operated on between 1950 and 1954. It is probable, therefore, that they were refugees with actual residence in Lebanon since 1948. The distribution of the cases among Lebanese by year of operation is shown in Figure 1. The average number of cases per year among Lebanese from 1949 to 1959 was 63.

Cases of Hydatid Disease among Lebanese

Surgical cases of hydatid disease occurred in patients from each of the governorates and from

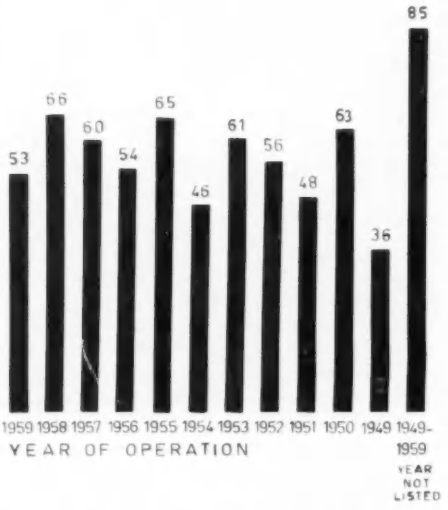

FIG. 1. Numbers of surgical cases of hydatid disease among Lebanese by year of operation, 1949 to 1959.

FIG. 2. Numbers of surgical cases of hydatid disease among Lebanese, 1949 to 1959, by district of residence.

all but one of the districts (Figure 2). The annual case rate for Lebanon for 1949 to 1959, based upon the officially registered population (1959), was 3.82 per 100,000 population. Average rates for each of the governorates and districts,

based upon uncorrected official populations (1959), were: North Lebanon 3.35 ('Akkar 2.01, Tripoli 4.48, Zgharta 0.68, Kura 1.99, Batrun 3.11); Mount Lebanon 3.58 (Kisrawan 2.16, Matn 6.94, B'abda 3.26, 'Aley 4.03, Shuf 1.85); Beirut 8.29; South Lebanon 1.19 (Jizzin 0.79, Sidon 1.85, Tyre 0.26, Marj'uyun 1.87); and Beqa' 3.65 (Hermil 0.00, Ba'albek 3.98, Beqa' 4.54, Rashayya 0.43).

There was virtually no difference in number of cases between males and females although, according to both Churchill[2] and Gholl,[4] males are more numerous in the population. A disproportionately high number of cases occurred in Christians as compared to Moslems and in Armenians as compared to Arabs (Figure 3).

Distribution of cases by age at operation is shown in Figure 4. Age specific rates could be calculated for Beirut only; these are given in Table 3.

Occupation was recorded for only 124 of the 738 patients. Forty-one females were identified as housewives and 15 of the patients as students. Of 338 Lebanese males, nonspecific occupation (laborer, worker, employee) was listed for 19. For only 49 males were specific occupations recorded; of these, 7 were shoemakers, 7 shopkeepers, 6 soldiers and 5 carpenters or plasterers.

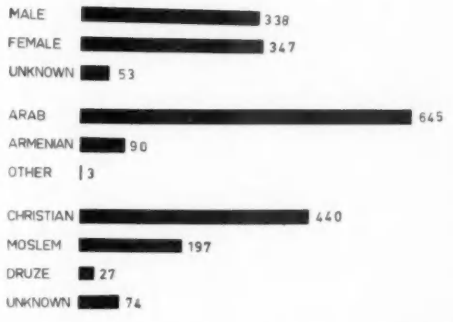

FIG. 3. Total numbers of disclosed surgical cases of hydatid disease among Lebanese by sex, religion and ethnic origin of patient.

FIG. 4. Numbers of surgical cases of hydatid disease among Lebanese, 1949 to 1959, by age of patient at operation.

TABLE 3

Age-specific rates for surgical cases of hydatid disease in the Beirut governorate, 1949 to 1959

Age at operation (yr)	Cases/year	Cases/year/100,000 population*
0–9	0.27	0.33
10–19	2.00	2.38
20–29	5.73	9.24
30–39	3.73	9.82
40–49	3.55	9.59
50–59	2.36	9.22
60–69	0.36	2.77
70+	0.27	3.86

* Calculated upon the 1959 official registered population of Beirut, corrected by a factor of 1.35 and distributed by age according to Churchill.[2] A 1954 or mid-point population would have given slightly higher rates.

Of the 668 Lebanese cases for which organ was specified, 365 or 54.6% involved the liver, 219 or 32.8% the lungs, 17 or 2.5% the kidneys, 15 or 2.2% the spleen, 3 or 0.5% the brain and 49 or 7.3% other organs. Organ was not specified for 70 cases.

Results of a Casoni skin test were recorded for 145 cases. Of these, 93 or 64% were positive or doubtful. For 163 cases results of the Weinberg complement fixation test were recorded. Ninety-three or 57% of these were positive or doubtful. The results of the hemagglutination test of Garabedian *et al.*[13] were recorded for 34 cases, of which 25 or 71% were positive or doubtful.

DISCUSSION

Hospital records are the only source of data on human hydatid disease in Lebanon at the present time. Because echinococcosis is not a reportable disease, the objective of this study was the disclosure of surgically confirmed cases in Lebanon for each year of the past 11-year period. In the absence of a case-finding procedure suitable for the mass screening of populations it can only be guessed as to what portion of existing human hydatid disease is revealed by a survey such as this. Large autopsy series are not available for

study in Lebanon, because, for cultural reasons, permission for autopsy is infrequently given. (The autopsy rate for 1959 at the American University Hospital, exclusive of stillbirths, was 11.7%.) These data, therefore, indicate a minimum level of infection in the population.

Since incorrect diagnosis in surgical cases of hydatid disease is rare, the number of revealed cases should be reliable. Errors are undoubtedly present in data concerned with residence of patients, however. The patient, in some instances, may have given the nearby address of a relative while even the long-time resident of a locality may have given his native or family village when asked for his address.

One of the most interesting findings of this survey was a higher incidence of hydatid disease among Christians than among Moslems. Assuming an equal number of Christians and Moslems in the population, the average annual case rate for Christians would be 5.00, and that for Moslems 2.24, per 100,000 population (calculated upon the official population estimate for 1959). It is tempting to ascribe this difference to cultural practices.* These would lead one to presume that Lebanese Moslems are less likely to have contact with dogs than are Lebanese Christians. An effort was made to ascertain whether or not this were true.

Although there is little information available on the extent to which dogs are kept for work or as pets in the Middle East, it has been a common observation of foreigners that Lebanese adults, whether Moslem or Christian, rarely display the interest in or fondness for dogs shown by northern Europeans or Americans. Numbers of feral or semi-feral dogs do roam many of the towns, however. In recent years, the American University Medical School has purchased annually 700 to 900 stray dogs from Beirut and its environs. Hunting dogs and watchdogs are owned by some Lebanese villagers and townspeople and both husky-type and saluki-type dogs are kept as herd dogs or watchdogs by Bedouins.

Some information on religion as a possible epidemiological factor was obtained through a questionnaire submitted to 96 University students from this area. This group consisted of 56 Christians, 35 Moslems and 5 Druzes. In reply to a question, "Has your family owned a dog within your memory?" 54% of the Christians and 46% of the Moslems answered "yes." To the question, "Do you recall ever playing with dogs as a child?" 66% of the Christians and 34% of the Moslems answered "yes." When asked if they would now ordinarily handle a dog, 89% of the Christians and 49% of the Moslems replied "yes." This sample of University students was, of course, not representative of the general population.

In 1959, during mass Casoni-testing of residents of Talabayya, a mixed Moslem and Christian farming village in the Beqa' district, 184 consecutive school children tested were asked to list the species of domestic animals owned by their families. Families of 27 of the 163 Christian children (16.6%), but only 1 of the 21 Moslem children (4.8%), owned dogs.

A third indication of the likelihood of contact with dogs was obtained from the records of the Rabies Center of the Lebanese Ministry of Health. Through the co-operation of the Director, Dr. Robert Abdo Hanna, the names, addresses and ages were obtained of all persons receiving prophylactic anti-rabic treatment during 1958 and 1959, as well as the species of animal responsible for the bite in each instance. Although religion was not recorded as such it could be ascertained from the patients' names for 308 of 361 individuals in 1958 and for 484 of 536 individuals in 1959. This Center is the only one in Lebanon where anti-rabic treatment is available. Treatment is administered free of charge and the individuals treated were from all parts of the country. An approximately equal number of Moslems and Christians received treatment each year for rat bites, while Christians predominated in the case of dog bites. Of patients 10 years of age or less who were bitten by dogs, the numbers of Moslems and Christians were approximately equal, while for older age groups Christians predominated (Table 4).

One question which arose with regard to religion as an epidemiological factor was that of the

* From the *Hadith*, which is surpassed only by the *Koran* as the source of authority for Moslem canon law:

"If a dog drinks from your vessel, you must wash the vessel seven times,"

"Angels do not enter a house where there is a dog,"

"The one who owns a dog which he is not able to replace by planting or by cattle, his production decreases everyday by one *hirat* (inch),"

"The Prophet has ordered the killing of dogs, except for a hunting dog or a sheep or cattle dog."

These quotations from the *Hadith*, as compiled by al-Bukhari (810–870 A.D.), were very kindly supplied by Mr. Abdul Azziz al-Duri, Visiting Professor of Arab History.

TABLE 4

Patients given prophylactic anti-rabic treatment for dog bite, by age and religion, 1958 to 1959, Rabies Center, Lebanese Ministry of Health

Age groups (yr)	Christians		Moslems	
	No.	%	No.	%
0–10	79	24.0	74	41.7
11–20	92	28.0	43	24.3
21–30	56	17.0	26	14.7
31–40	40	12.2	13	7.4
41–50	35	10.6	9	5.1
51–60	19	5.8	5	2.8
61+	8	2.4	7	4.0
Total.....	329	100.0	177	100.0

availability of medical attention to the different segments of the Lebanese population. Such information is not really known and its influence upon these findings, therefore, remains obscure. With approximately 1 physician per 1,318 persons and 1 hospital bed per 282 persons, total medical facilities for Lebanon would appear to be relatively good. Although physicians and hospitals are located in most of the larger towns, much of the physician population is concentrated in Beirut: total physicians in Lebanon (1955), 1,204;[5] in Beirut (1959), 840.[14] The fact that few surgical cases of hydatid disease were disclosed by this survey among residents of Tyre and Hermil, two economically depressed rural areas, suggests the relevance of this question. Characteristics shared in common by Tyre and Hermil are their relatively dry climates and preponderant Shiite Moslem populations.

The higher rate of infection with hydatid disease among Christians was noted for the city of Beirut as well as for the other governorates. This in itself suggested that religion was a real epidemiological factor among Beirutis at least, since both the government and voluntary agencies in the city maintain inexpensive or free medical services.* Evidence of this was obtained by a comparison made between the religious affiliations of total admissions and of hydatid disease patients at the 230-bed Beirut Government Hospital. This hospital is located in the Mudawwar, the most economically depressed quarter of Beirut, which, with adjacent Burj Hammud, accounted for a large number of the hydatid disease cases. The out-of-date municipal slaughter house is located in this quarter. For the 18-month period of June, 1958, to December, 1959, there were 2,447 total admissions, 45% of whom were Christians and 49% Moslems. Of the 23 patients with hydatid disease operated upon in this hospital from 1949 to 1959, however, 70% were Christians and 30% Moslems.

These additional data tend to confirm the greater risk of infection with hydatid disease among Christians as compared with Moslems in Lebanon. The accessibility of medical attention in poorer and more isolated areas of the country remains an uncertain factor, although Powers *et al.*[16] have demonstrated a correlation between infant mortality rates and relative isolation of Lebanese villages. With some exceptions, isolated areas are not exclusively Moslem or Christian, however.

The much higher case rate for Lebanese-Armenians than for the general population is another point of interest. Assuming a present Armenian population of 100,000 (see Table 3), the average annual incidence among Armenians would be 8.18 per 100,000, over two times that for the general population and one of the highest rates reported. Several possible reasons for this unusually high rate of infection suggest themselves, but as yet they have not been explored sufficiently.

Age specific rates could be calculated for Beirut governorate only. Their value is questionable since the incubation period for hydatid disease, though generally prolonged, is of variable duration. One may observe merely that operations for hydatid disease in Lebanon occurred with approximately the same frequency among individuals between 20 and 60 years of age.

The anatomical locus of the cyst in the patient has no known epidemiological significance. It is of interest to note, however, that hydatid cysts of the lungs were found to be of relatively more frequent occurrence among Lebanese than has been reported by many earlier investigators for other populations. It is possible that this may re-

* Lebanon has a large number of voluntary welfare agencies. In Beirut alone, in 1958, there were 11 nongovernmental charitable societies concerned primarily with medical services (of which 7 were nonsectarian, 1 Sunni Moslem and 3 Christian) and 26 societies concerned primarily with welfare and relief (of which 6 were nonsectarian, 3 Sunni Moslem, 14 Christian and 3 Druze). Beirut nongovernmental welfare agencies employed in that year 287 physicians and 57 nurses on either a paid or voluntary basis.[15]

flect a relative awareness of chest disease on the part of Lebanese and/or the fact that there is a concentration in Lebanon of chest specialists and sanatoria which serve the entire Middle Eastern area. On the other hand, the distribution by anatomical site reported recently by Foster[17] for New Zealand does not differ substantially from that observed here.

From the standpoint of completeness, occupational data left much to be desired. The occupation of the patient was seldom ascertained by the physician in taking the patient's history. Though these data were limited, the number of cases of hydatid disease among shoemakers was striking. According to hospital records in which specific occupation of males was given, 14% of the patients were shoemakers. How many of these individuals made shoes and how many also repaired shoes is not presently known. The work of the latter group, spent in handling and remaking used street shoes, might place them in a position of considerable risk. Work habits of shoemakers (*e.g.*, placing tacks in the mouth and moistening thread with the lips) would presumably contribute to the risk of exposure. Unfortunately statistics for Lebanon are not available on the distribution of the population by occupation.*

These data, while necessarily deficient in a number of respects, show that echinococcosis is a disease of major public health importance in Lebanon. They should provide some baseline for future estimates. For comparison, recent hydatid disease annual rates per 100,000 population for other countries with known high levels of infection include Argentina (1956 to 1958) 0.71, Chile (1956 to 1958) 1.69, Uruguay (1956 to 1958) 2.37,[18] Cyprus (1936 to 1949) 9.56[19] and New Zealand 4.46.[17] Rates for Argentina, Chile and Uruguay were calculated upon 1957 estimates of population,[8] that for New Zealand upon the 1951 estimate.[20] Lebanon's rate of 3.82 is among the highest reported.

SUMMARY

The average number of surgical cases of hydatid disease per year among Lebanese during 1949 to 1959 was 63. The case rate was 3.82 per 100,000 population per year. The heaviest concentration of cases was in Beirut and environs. Case rates were much higher among Christians (5.00) than among Moslems (2.24) and among Armenians (8.18) than among the general population. Age specific rates for age at operation for patients from Beirut showed that operations were performed with equal frequency among individuals 20 to 60 years of age. An unusually high percentage of infected males were shoemakers.

* After the manuscript went to press we learned that, in leather manufacture, hides are subjected, before tanning, to a softening process called "bateing" or "puering." This is ordinarily done by immersing the hides in dog or bird feces at 37°C, although more purified enzyme products have supplanted the use of feces in some countries. The extent to which dog feces are now used for bateing hides in Lebanon remains to be determined.

ACKNOWLEDGMENTS

We are grateful to Messrs. Vazken Der Kaloustian, Mtanios Koussa and Elias Matouk for their assistance and to a number of co-operating physicians, but in particular to Drs. Raymond Johanny and Denis Ciaudo, Hotel Dieu of the Faculté Française de Médecine; Papken Mugrditchian, Azounieh Sanatorium; Wahib Nini, Nini Hospital, Tripoli; Georges Azar, Kennedy Memorial Hospital, Tripoli; Joseph Daoud, Government Hospital, Zahle; Elias Khoury, Bhannes Sanatorium; and Yervant Jidejian, American University of Beirut. Dr. Charles W. Churchill very kindly reviewed the manuscript.

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SIDE EFFECTS OF CIBA-1906

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The drug, Ciba-1906, has been used in the treatment of patients with leprosy for some time and it appears that no reactions have been reported. However, a number of reactions have occurred from the use of this drug in patients who do not have leprosy or tuberculosis.

Several years ago a systematic study was undertaken with Ciba-1906* in the treatment of various dermatologic disorders. The series included 286 patients with rosacea, dermatitis herpetiformis, pemphigus, psoriasis, nodular cystic acne, scleroderma, mycotic granulomata, sarcoidosis and lupus vulgaris. The dose range was 1 to 3 grams daily, with an average dose of 1.5 grams. The average duration of treatment was approximately 6 weeks.

During the initial period of study no reactions of any sort were recorded.[1] In recent months eight generalized morbilliform eruptions associated with fever were observed in 68 patients. These were not severe and usually occurred in patients irrespective of the skin disorder treated. The dosage schedule ranged from 1.5 to 2 grams a day. The rashes usually occurred within the 2nd week of therapy and subsided promptly when the drug was discontinued. In one patient a test dosage of 500 mg was given some 2 weeks after the eruption had subsided and there was a prompt recurrence. We have noted also occasional diarrhea, nausea, and bad taste in the mouth. In a series of eight patients with aphthous stomatitis and oral pemphigus, the tablets were sucked for their local effect; these patients complained about the bad taste in the mouth. We have also received information[2] about several other cases of drug rash and diarrhea and bad taste in the mouth. No severe blood dyscrasia nor other visceral toxicity, such as hepatitis, was observed in the present series.

It appears that, although the drug was not used as extensively in this study as it has been used in the treatment for leprosy, a significant number of drug reactions did occur. These were essentially of the minor drug-allergy type.

* Furnished by Dr. Harold J. Bornhold, Division of Clinical Investigation, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

SUMMARY

The reactions noted in a series of 286 patients treated with Ciba-1906 for various dermatologic conditions were mild and subsided after the drug was discontinued. In one patient the eruption recurred promptly following a test dose. Nausea, diarrhea and a bad taste in the mouth were other reactions noted. Drug allergy should be considered when morbilliform eruptions appear in patients under Ciba-1906 therapy.

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NDUMU VIRUS, A HITHERTO UNKNOWN AGENT, ISOLATED FROM CULICINE MOSQUITOES COLLECTED IN NORTHERN NATAL, UNION OF SOUTH AFRICA*

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Since January, 1956, a station located near Ndumu in northern Natal has been the base for periodic field activities concerned with the study of arthropod-borne viruses. The scope of this program and the basis for selection of the site have been referred to by Kokernot *et al.*[1]

The purpose of this paper is to record the isolation of two strains of a hitherto unknown virus from mosquitoes collected near the field station. It is proposed that the agent be named Ndumu virus.

MATERIALS AND METHODS

Previous reports from this laboratory have described the methods utilized in capturing mosquitoes[2] and processing them for possible virus isolation.[3] At the field station mosquitoes are pooled by species, killed with cyanide[4] and then transferred to sterile Wasserman tubes which are plugged with wax-coated corks. The tubes are then stored in a cabinet or thermos flask containing CO_2 ice until they are processed at the laboratory in Johannesburg.

The techniques for the preparation of antigens and mouse hyperimmune serum and the methods for doing complement-fixation (CF) and hemagglutination-inhibition (HI) tests have been described.[5, 6] Neutralization (NT) tests were done according to the methods described by Smithburn[7] and Smithburn *et al.*[8]

The technique used to demonstrate the sensitivity of Ndumu virus to sodium desoxycholate was essentially that described by Theiler[9] with only a slight modification. The diluent used in these studies was a 0.2% solution of bovine plasma albumin (BPA) rather than the 0.75% adopted by Theiler. Virus dilutions were made before exposure to the chemical.

* The studies and observations on which this paper is based were financed jointly by the South African Institute for Medical Research, the Poliomyelitis Research Foundation, the South African Council for Scientific and Industrial Research and The Rockefeller Foundation.

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RESULTS

One of the virus strains was isolated from 19 *Mansonia* (*Mansonioides*) *uniformis* (Theobald) collected May 7, 1959. The lot number given this pool of mosquitoes was AR 2204. They were processed on May 19, 1959, and inoculated into two litters of newborn mice. All mice were sick on the 6th postinoculation day; two were sacrificed for subinoculation. The agent was readily established in adult mice by intracerebral inoculation of 1st passage infected infant mouse brain material.

AR 2204 virus was successfully reisolated from newborn mice inoculated with the original mosquito suspension 6 days after the primary inoculation.

A 10^{-2} virus dilution in 10% broth failed to pass through a Seitz EK pad, but was filterable through a gradocol membrane with an APD of 275 mμ.

The second virus strain was isolated from a pool, designated AR 2211, of 278 *Aedes* (*Neomelaniconion*) *circumluteolus* (Theobald) collected near Ndumu on May 5, 1959. They were processed and inoculated into two litters of newborn mice on May 19. The results in one litter were unsatisfactory. In the other litter one mouse was sick on the 7th postinoculation day; it was sacrificed for passage and a serially transmissible agent established in both newborn and adult mice. The remaining mice in the second litter were dead or were sick and sacrificed for further study between the 11th and 16th postinoculation days.

The results of an attempted reisolation done on May 26 were inconclusive.

AR 2211 virus was filterable through a gradocol membrane (APD 275 mμ), but it failed to pass through a Seitz EK pad.

Immunological Studies

The results of a cross CF test are shown in Table 1 and the results of a cross NT test, in

TABLE 1

Results of cross complement-fixation tests with AR 2204 and AR 2211 virus antigens

Antigen		Mouse hyperimmune serum*									
		AR 2204, 1 in					AR 2211, 1 in				
		8	16	32	64	128	8	16	32	64	128
AR 2204 1 in	8	4	4	4	3	0	4	4	3	3	0
	16	4	4	4	3	0	4	4	3	1	0
	32	4	4	2	0	0	4	4	3	1	0
	64	3	2	0	0	0	3	3	2	1	0
	128	0	0	0	0	0	0	0	0	0	0
AR 2211 1 in	8	4	4	4	3	0	4	4	4	0	0
	16	4	4	4	3	0	4	4	4	0	0
	32	4	3	2	0	0	4	4	3	2	0
	64	2	0	0	0	0	4	4	3	2	0
	128	0	0	0	0	0	2	0	0	0	0

* Serum control negative.

TABLE 2

Results of cross intracerebral adult mouse neutralization tests with AR 2204 and AR 2211 virus strains

Serum*	Virus strain, logs neutralized	
	AR 2204	AR 2211
AR 2204	2.4	4.6
AR 2211	2.9	3.7

* Pre- and postinoculation guinea pig sera for the respective virus strain were used.

Table 2. These two serological tests demonstrate that AR 2204 and AR 2211 viruses are identical.

An antigen for AR 2204 strain was tested in three dilutions (1:4, 1:8 and 1:16) in a screening CF test against a 1:4 dilution of the following mouse hyperimmune antisera: Sindbis, Chikungunya, Semliki Forest, Middelburg, Mayaro, Wesselsbron, Spondweni, West Nile, H 336, Uganda S, dengue 1, dengue 2, yellow fever, Zika, Ntaya, Bunyamwera, Germiston, Oriboca, Simbu, Rift Valley fever, Pongola, Bwamba fever, Lumbo, Mossuril, AR 344* and AR 1776.‡ The results of this test were negative.

No HA mouse-brain antigen has been obtained for AR 2204 or AR 2211 virus strain by alkaline aqueous, acetone-ether or sucrose-acetone technique. The level of viremia (AR 2211 virus) in pooled infant mouse sera was $10^{-4.4}$ LD_{50} as determined by a titration of the sera using adult mice inoculated intracerebrally. Accordingly, no attempt was made to obtain an HA antigen from mouse serum.

However, AR 2204 and AR 2211 mouse hyperimmune sera prepared by giving adult mice four intraperitoneal inoculations (0.1 ml each) of a 10% suspension of virus-infected brain material were tested in 1:20 dilutions in an HI test against the following antigens: Sindbis, Chikungunya, Semliki Forest, Middelburg, Wesselsbron, Spondweni, West Nile, H 336, dengue 1, dengue 2, yellow fever, Oriboca, Germiston, Bunyamwera and Rift Valley fever. The results of this test were negative.

AR 2211 virus was not neutralized in an intracerebral infant mouse NT test by potent antisera against the following viruses: Pongola, Bwamba fever, Wesselsbron, Spondweni, West Nile, H 336 and AR 1776. In an intracerebral adult mouse NT test, Bunyamwera virus and Simbu virus antisera also failed to neutralize the agent. With the exception of Bwamba fever virus, strains of each of these agents have been isolated from specimens collected at Ndumu.

Thus, immunological studies utilizing three serological techniques have provided no evidence that Ndumu virus is related to any of the agents against which it has been tested.

Physical and Chemical Properties

A suspension of Ndumu virus using as diluent 5% lactose, 1% peptone M/25 phosphate buffer in water, withstands lyophilization without loss in titer. Such a stock virus preparation titered $10^{-6.4}$ prior to 24-hour freeze-drying and $10^{-6.5}$ afterward.

Ndumu virus is sensitive to the action of sodium desoxycholate. The virus titer in the control titration was $10^{-5.6}$ while the virus titer after contact with sodium desoxycholate was $10^{-1.6}$, a difference of 4 logs.

Histopathology

The lesions produced by intracerebral inoculation of newborn and adult mice with Ndumu virus are limited to central nervous system tissue. These lesions are typical of a viral encephalitis and are characterized by generalized, round

* Viruses isolated by this Unit but not yet identified.

cell infiltration in the perivascular tissue with scattered foci of degenerating and necrotic cells.

Pathogenicity for Laboratory Animals

Mice. Both newborn and adult mice are susceptible to intracerebral inoculation of Ndumu virus. The average survival time in younger mice is about 3 days and in older mice, about 7.5 days. In newborn mice results of titrations of the virus by intracerebral and intraperitoneal inoculation were about the same. However, when titrated intracerebrally and intraperitoneally in adult mice the end-points were $10^{-7.5}$ and less than $10^{-2.0}$, respectively; thus the pathogenicity for adult mice by peripheral inoculation is quite low.

Guinea pigs. Two groups of eight guinea pigs each were inoculated intracerebrally with the two strains of Ndumu virus. One group received 870 adult mouse IC LD_{50} of AR 2204 virus and the other group received about 1,300 LD_{50} of AR 2211 virus. None of the individuals in either group developed signs of illness, but pooled postinoculation sera for each neutralized a significant amount of the homologous virus.

Monkey. A nonimmune adult *Cercopithecus aethiops pygerythrus* F. Cuvier was inoculated intracerebrally with 4,000 adult mouse IC LD_{50}. Serum obtained from this monkey on post-inoculation days 2, 5, 7, 8, 9 and 10 was inoculated intracerebrally into newborn mice in an attempt to detect a viremia. On days 8 and 10, from the brain of a sick mouse in each of the respective mouse groups, an agent was isolated which was shown to be Ndumu virus by NT test using adult mice. In spite of the viremia, however, the monkey showed no signs of illness. Sera taken 12 and 29 days postinoculation failed to neutralize the homologous virus.

On the 29th postinoculation day, the monkey was given a booster inoculation of Ndumu virus, 51,000 adult mouse IC LD_{50} intramuscularly and 102,000 LD_{50} intraperitoneally. A specimen taken 29 days later neutralized 4.2 logs of the virus in an adult mouse NT test.

Protection Test Survey

Ndumu virus was tested against sera from residents of eight widely scattered localities in southern Africa. In these tests adult mice were inoculated intracerebrally.

From the results shown in Table 3, it is apparent that Ndumu virus or some closely related agent has a wide distribution in southern Africa. Although the number of donors by age group for each locality is small, the evidence of activity in the younger group suggests that the agent might be endemic.

TABLE 3

Results of intracerebral adult mouse neutralization tests with Ndumu virus against sera from residents of eight widely scattered localities in southern Africa

Locality by region	Ratio by age group*	
	Children	Adults
Mozambique		
Lumbo	ND†	0/20
Namacurra	ND	3/16
Nova Lusitania	1/15	1/15
Northern Natal		
Ndumu	1/10	9/44
Lake Simbu	1/12	1/20
Bechuanaland		
Seronga	1/30	ND
Gomare	0/11	2/19
Nokaneng	1/10	1/19

* In the ratios the numerator refers to the number of protective sera and the denominator, the number of sera tested.

† No tests done.

DISCUSSION

Tongaland, the region of Natal in which Ndumu is located, has ecological features favorable to the activity of arthropod-borne viruses,[10] and a major objective of the field program conducted there has been the search for new agents in this category. During the past 4 years multiple strains of several known viruses have been isolated. Continued efforts, however, have been rewarded by the discovery of new virus types, as exemplified by Ndumu virus.

The epidemiology and epizootiology of these virus isolates remain obscure. Their detection at Ndumu during the course of fairly intensive field work has been sporadic with respect to distinct virus types. In the discussion which followed the presentation of the paper by Smithburn and de Meillon[11] at the Sixth International Congresses on Tropical Medicine and Malaria, it was suggested that the sporadic nature of the activity was due, in effect, to a dynamic focus

of virus activity moving about in a larger ecological zone, and that in the wake of such foci favorable niches were left as seed beds for future bursts of similar activity.

In an analysis of the mosquito collections made at Ndumu and subsequent virus isolations, Worth *et al.*[12] discuss this problem in detail.

SUMMARY

A virus strain (AR 2204) was isolated from a pool of 19 *Mansonia* (*Mansonioides*) *uniformis* (Theobald); another virus strain (AR 2211) was isolated from a pool of 278 *Aedes* (*Neomelaniconion*) *circumluteolus* (Theobald).

Serological studies utilizing CF and NT techniques have shown the two virus strains to be immunologically indistinguishable. Similar studies including also the HI technique indicate that the virus is not related to a wide spectrum of other arthropod-borne viruses. The agent has been named Ndumu virus after the locality where the mosquitoes were collected.

Ndumu virus is sensitive to the action of sodium desoxycholate. A vervet monkey was inoculated with Ndumu virus and the agent was detected in the serum on the 8th and 10th postinoculation days. Protection test surveys indicate that Ndumu virus has been active among human beings residing in widely scattered areas in southern Africa.

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ST. LOUIS ENCEPHALITIS IN PANAMA

II. Survey of Human Blood for Antibodies against St. Louis and Two Related Group B Viruses, Ilhéus and Yellow Fever*

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In 1958 a focus of infection with the virus of St. Louis encephalitis (SLE) was discovered in the Republic of Panama in the Province of Darién on the Atlantic slopes of the Continental Divide near the Colombian border.[1] The virus was repeatedly recovered from mosquitoes or human blood over an 8-month period from February, 1958, when stations for the collection of canopy mosquitoes were first established, to September of the same year, when they were discontinued. Further epidemiological studies were planned to determine the immunological status of the local population and possible animal reservoirs. In the present report results of a survey of blood drawn from persons resident in the area for antibodies against SLE as well as against two other group B viruses, yellow fever and Ilhéus, are presented. All three viruses are known to be active at least sporadically in Panama. The virus of Ilhéus encephalitis was isolated from mosquitoes captured at the collecting station at Paya, Darién, in June 1958,[2] and thus was simultaneously present with SLE. No evidence whatsoever of recent activity of yellow fever was obtained. However, jungle yellow fever occurred in this section of the Republic in 1948 to 1949 and again in 1956 to 1957. As several vaccination campaigns have been conducted, of which complete records are not available, no attempt is made to differentiate between a positive serology as a result of artificial immunization to yellow fever and natural infection.

Topographical and other ecological data. The study was conducted among the human population occupying the portion of the Tuira River which extends between the mouths of the Pucro and Paya tributaries as well as in two small communities located well up these last two rivers. The area is mainly covered by forest of an intermediate type between tropical rain forest and tropical deciduous forest, with occasional clearings occupied by human habitations and by small banana and plantain patches. Rainfall is concentrated between the months of May and December, with only intermittent and light precipitation during the dry season which extends from January through April. The human population is made up of a mixture of Colombian Negro immigrants and Choco and Cuna Indians. The Cunas are congregated in two small stable communities located well up the Pucro and Paya Rivers, while the Colombian Negroes and Choco Indians are mostly nomadic, living in temporary houses located along the Paya and Tuira Rivers.

MATERIALS AND METHODS

A total of 195 blood specimens was collected in December, 1958, from all available inhabitants of the small villages and settlements along the Tuira, Pucro and Paya Rivers. They were packed in ice and transported by boat and plane to the laboratory located in Panama City where the sera were removed and stored until tested. Slightly more males (105 or 54%) than females (90 or 46%) were represented in the survey. Ages varied from 1 to 70 years and the length of residence in the area, from 8 days to 70 years.

For the neutralization tests aliquots of pure serum and appropriate viral dilutions were incubated for 2 hours at 37°C, chilled and injected intracerebrally into groups of 6 mice. A virus titration and negative and positive controls were included. In testing for antibodies to SLE virus a preliminary screening test was conducted using two serial dilutions of virus as recommended by Hammon,[3] the first containing 10 to 25 LD_{50} per inoculum and the second, 10 times this concentration. Sera giving a positive reaction in the screening test were titrated against higher serial concentrations of virus. In the reactions with Ilhéus virus each serum was tested against two serial dilutions of virus also, but in this case the

* This investigation was supported by research grant E-1941 from the National Institutes of Health, U. S. Public Health Service.

first dilution was calculated to contain 50 to 75 LD_{50} per inoculum and the second, 10 times this concentration, no further titration being conducted. For determining antibody response to yellow fever, a single virus dilution containing approximately 100 LD_{50} of virus was used, a 6/6, 5/6, 5/5, 4/5 or 4/4 survival ratio being interpreted as positive.

Virus strains used as antigens in the neutralization studies were as follows: SLE—a local strain isolated from *Sabethes chloropterus* captured at Buena Vista in 1957;[1] Ilhéus—a strain isolated from a mixed pool of mosquitoes of the genus *Psorophora* captured in Honduras in 1954;[4] and yellow fever—the French neuroadapted strain received through the courtesy of Dr. Max Theiler of The Rockefeller Foundation Virus Laboratories.

RESULTS

Sixty-nine, or 31%, of the sera neutralized 1 log or more of SLE virus; 27, or 13.8%, neutralized 1.5 logs or more; and 16, or 8.4%, 2 or more logs. Seven persons resident in the area for 20 to 70 years had titers of 3 or more logs of virus neutralized. Eighty-five, or 43.7%, of the sera neutralized 2 or more logs of Ilhéus virus and 82, or 42%, gave a positive reaction against 2 or more logs of yellow fever virus. Another 21 (11%) sera showed a doubtful reaction against the latter virus. For purposes of tabulation and comparison (see Table 1) sera neutralizing 2 or more logs of virus have been classified as positive. It is recognized that this figure does not take into account weak antibody responses, falling titers due to the passage of time since infection was incurred, or heterologous reactions. Most of the sera neutralized more than one virus. However, it may be considered significant that 4 were single positives against SLE virus, 28 against Ilhéus and 29 against yellow fever. Seventy-one sera were negative against all three. With all three viruses the proportion of positive reactors among males was higher than among females. Although males constituted 53% of the persons tested, 62.5%, 65.8% and 56.1% of the sera neutralizing at least 2 logs of SLE, Ilhéus and yellow fever viruses, respectively, were from males. Age also seemed to be a significant factor in the incidence of infection. Although 45 children under 10 years of age, or 23% of the total, were included in the survey, only 2, or 4.5%, were positive to SLE and 3, or 6.7%, to Ilhéus. Positives against all three viruses were scattered throughout the area surveyed and not significantly concentrated in any one place. Since the viruses of SLE and Ilhéus encephalitis were isolated from local mosquitoes shortly prior to the collection of blood samples, it is not known whether the presence of neutralizing antibodies was the result of recent or old infection. No data were gathered concerning illnesses suffered prior to the survey.

TABLE 1

Results of neutralization tests against three group B viruses with the blood of persons residing in a rural area of Darién

Virus	No. and % positive*
St. Louis	16 (8.4%)
Ilhéus	85 (43.7%)
Yellow fever	82 (42.0%)
Virus neutralized	**No. of sera neutralizing**
St. Louis only	7
Ilhéus only	28
Yellow fever only	29
SLE and Ilhéus	7
SLE and YF	3
Ilhéus and YF	40
SLE, Ilhéus and YF	10
None	71
Total	195

* Sera neutralizing 2 or more logs of virus.

DISCUSSION

Interpretation of results is complicated by the fact that the three viruses are antigenically related and may show varying degrees of cross-reaction. Immunological overlap among group B viruses has been amply demonstrated.[5-7] Theiler and Casals[8] have recently shown that the production of heterologous antibodies is greatly stimulated by infection with a second group B virus after recovery from a previous infection with another member of the same group. They conclude that no single serological test will permit accurate diagnosis of such secondary infections. Furthermore, although we

have demonstrated the presence, at least sporadically, of three group B viruses in Panama, there is no reason to believe that others may not occur. In fact a number of other as yet unclassified viruses were isolated in the course of this work.

As regards SLE infection in Darién, the picture is relatively clear. Virus was isolated repeatedly from mosquitoes and from the blood of two persons who were especially exposed to their bites. Also about 8% of persons residing in the study area had high titers of antibodies in their blood. In this forested area the disease apparently takes a relatively mild form and is transmitted to man by canopy mosquitoes. Like sylvan yellow fever it is more common in males old enough to engage in field work. No urban outbreaks of the disease have been recognized.

As regards Ilhéus infection much information is still lacking. The virus was isolated once from arboreal mosquitoes in Paya, Darién; and among the local population there is a surprisingly high percentage of persons with neutralizing antibodies in their blood (43.7%). However, the infection has never been recognized clinically nor has the virus been isolated from a human being in Panama. Obviously more extensive investigation is required.

In the case of yellow fever no differentiation can be made between persons immune as a result of vaccination and as a result of natural infection. There were 82, or 42%, positive and an additional 21, or 11%, doubtful reactions against 2 logs of virus. These figures indicate that only about half of the rural population in the area is demonstrably protected against yellow fever although this is the group most exposed to danger from the jungle form of the disease. Results very similar in certain respects to ours have been obtained by Causey and Theiler[9] in Brazil and by Groot and co-workers[10] in Colombia, indicating similar epidemiological patterns for these viruses in tropical America.

SUMMARY

One hundred and ninety-five persons residing in a forested area of the Province of Darién in Panama near the Colombian border were tested for neutralizing antibodies against yellow fever, St. Louis encephalitis (SLE) and Ilhéus viruses. Capacity to neutralize 2 or more logs of virus was observed in 8.4% against SLE, in 43.7% against Ilhéus and in 42% against yellow fever. The interpretation of these results is discussed.

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ST. LOUIS ENCEPHALITIS IN PANAMA

III. Investigation of Local Mammals and Birds as Possible Reservoir Hosts*

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In 1958 a focus of infection with the virus of St. Louis encephalitis (SLE) was encountered in the Province of Darién near the Colombian border of Panama. Details concerning the isolation of the virus and the immune status of the local population appeared in previous publications.[1, 2] The present report summarizes results of a survey of the fauna of the region for antibodies against SLE virus and attempts to recover virus from avian blood. Birds have been strongly incriminated in the epidemiology of this disease. The virus was isolated from natural infections in a nestling dove by Downs *et al.*[3] and in a flicker by Ranzenhofer *et al.*[4] Furthermore, high levels of serum antibody have been demonstrated in avian blood during field surveys[4-6] and experimental infections have been readily produced.[7-10]

MATERIALS AND METHODS

Blood samples were collected during a period of about 2 months from the end of February to the middle of April, 1959. Animals were obtained by shooting or trapping and were bled in the field by cardiac puncture. The blood specimens were then packed in ice and transported from Paya to El Real by boat and from El Real to Panama by plane.

The great majority arrived in satisfactory condition although a few were discarded because of excessive hemolysis. A total of 248 specimens was examined, 139 from mammals, 107 from birds and 2 from reptiles.

All were tested for neutralizing antibodies against SLE virus. The majority of those giving positive reactions to SLE were retested against yellow fever and Ilhéus viruses as an aid in the interpretation of the specificity of the result. The limiting factor was the quantity of serum available. In addition, 48 avian sera were injected intracerebrally into suckling mice for possible virus isolation.

* This investigation was supported in part by research grant E-1941 from the National Institutes of Health, U. S. Public Health Service.

Virus strains and techniques were as previously described,[2] except that preliminary screening of sera was performed with a single concentration of virus, 30 to 50 LD_{50} per inoculum. Where sufficient serum was available, specimens giving a positive reaction in the screening test were titrated against higher concentrations of virus.

RESULTS

Results of the neutralization tests are presented in Tables 1 and 2. Except for 2 of 39

TABLE 1

Results of mouse protection tests with vertebrate sera against St. Louis encephalitis virus

	Total no. tested	No. pos.
Herons	10	3
Chickens	22	0
Tinamous	2	0
Guans	6	1
Ducks	7	0
Cormorants	8	1
Hawks	10	1
Vultures	3	1
Parrots and macaws	4	0
Kingfishers	19	0
Toucans	6	3
Woodpeckers	3	0
Miscellaneous birds*	7	1
Bats	23	0
Marsupials	39	0
Spiny rats	39	2
Rats and mice	5	0
Pacas	3	0
Kinkajous	3	0
Monkeys	21	0
Miscellaneous animals†	8	0
Total	248	13

* Includes one anhinga, one rice grackle, one jay and one jacana.

† Includes one squirrel, one peccary, two agoutis, two anteaters, and two iguanas.

TABLE 2

Results of neutralization tests (NT) against yellow fever and Ilhéus viruses with sera positive to St. Louis encephalitis

Species	Total	No. pos. against SLE and titer*	Results of NT against	
			Yellow fever	Ilhéus
Cathartes aura (turkey vulture)	2	1 (2.2)	Neg.	0
Anhinga anhinga (water turkey)	1	1 (3.1)	Neg.	1.5
Phalocrocorax olivaceus (cormorant)	8	1 (<4.0)	Neg.	1.6
Nycticorax nycticorax (heron)	1	1 (at least 1.3)†	Neg.	1.5
Florida caerulea (heron)	6	1 (3.1)	Neg.	1.5
Butorides virescens (heron)	1	1 (at least 1.3)	Not tested	Not tested
Rhamphastos swainsonii (toucan)	2	2 (at least 1.3)	One neg., one pos.	One 1.5, one not tested
Rhamphastos sulfuratus (toucan)	3	1 (<4.0)	Pos.	0
Penelope cristata (guan)	5	1 (<4.0)	Pos.	Not tested
Unidentified Accipitridae (hawk)	2	1 (3.1)	Neg.	1.5
Proechimys semispinosus (spiny rat)	39	2 (2.0 and 1.7)	Pos.	Not tested

* Titer given as logs neutralized.

† Insufficient serum for further titration.

Proechimys semispinosus, no mammal gave a positive neutralization test against SLE virus. These two *Proechimys* were also weakly positive to yellow fever. Sera from 11 wild birds (10.3%) neutralized SLE virus, three of these in titer of more than 4 logs. Six also gave positive reactions to Ilhéus virus in low titer and three to yellow fever.

Forty-eight of the avian sera were injected intracerebrally into infant mice but no SLE virus was isolated. An as yet unidentified virus was recovered twice, however, once from the hawk, *Helicolestis hamatus*, and once from the woodpecker, *Dryocopus lineatus*. Birds tested were all adults. No blood specimens were obtained from fledglings.

DISCUSSION

Since SLE virus was not isolated from the blood of any of the vertebrates here described, it is impossible to affirm that the positive neutralization tests obtained against this virus were specific. It is of interest, however, that 6 of the 11 positive avian sera were single positives (if we disregard the low titers obtained against Ilhéus virus which, immunologically, is closely related to SLE). In view of the well established evidence incriminating birds in the epidemiology of SLE in other countries, it seems highly probable that they play an important role in Panama also. Contrary to the experiences of others, we did not find any positives to SLE virus among 22 chicken sera. Wild birds seem to be the preferred hosts in this area.

SUMMARY

Two hundred and forty-eight blood samples obtained from 139 mammals, 107 birds and 2 reptiles were tested for neutralizing antibodies against St. Louis encephalitis virus. Eleven, or 10.3%, of the avian specimens and two, or 1.4%, of the mammalian specimens neutralized this virus.

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ISOLATION OF THE VIRUS OF ILHÉUS ENCEPHALITIS FROM MOSQUITOES CAPTURED IN PANAMA*

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In 1956 isolation of the virus of Ilhéus encephalitis from mosquitoes of the genus *Psorophora* captured in Honduras was reported.[1] This represented the first isolation from Central America. The same virus was subsequently recovered from *Sabethes chloropterus* collected in Guatemala.[2] In the present publication we wish to report the first isolations from Panama of a virus identical with or very closely related to that of Ilhéus encephalitis. The virus was recovered twice from mosquitoes in 1958, once from *Haemagogus spegazzinii falco* collected at Cerro Azul in the Province of Panama and once from *Trichoprosopon spp.* collected at Paya in the Province of Darién.

MATERIALS AND METHODS

The isolations were effected in suckling mice inoculated intracerebrally with mosquito suspensions in accordance with techniques previously described.[3] Mosquitoes tested were sylvan arboreal species captured in glass vials as they alighted to feed on men stationed in platforms in the forest canopy and are not to be considered representative of the local culicine fauna as a whole. They were obtained from the two stations, Cerro Azul and Paya, which are described below.

Cerro Azul is a forested area about 60 km east of the Canal Zone near the headwaters of the Pacora River in the Province of Panama. Collections have been made here almost continuously from September, 1956, to the present time. From the time of initiation of the collections to July 1, 1960, a total of 34,643 mosquitoes have been tested including principally members of the genera *Haemagogus*, *Sabethes* and *Wyeomyia*. Although many other viral agents have been recovered in this location, only the one strain here described corresponds to Ilhéus virus.

Paya is located on the forested slopes at the junction of the Paya and Tuira Rivers near the Panama-Colombia border in the Province of Darién. Collections were made from February, 1958, to March, 1959, a total of 14,406 mosquitoes being tested. The most abundant genera were *Sabethes*, *Wyeomyia* and *Haemagogus* in that order. Only 278 *Trichoprosopon* were captured in all, from which the virus here reported was recovered.

The viral agents were identified by immunological comparison with the Honduran strain. Reliance was placed principally on neutralization tests using mouse hyperimmune sera and cross-immunity experiments in which mice immunized against the homologous strain were challenged with approximately 10,000 LD_{50} of the heterologous strain administered intracerebrally.

RESULTS

The strain of Ilhéus virus isolated from *Trichoprosopon spp.* was obtained from a pool of 37 of these mosquitoes collected at Paya over a period extending from April to June, 1958. Included in the pool were the following species: *Trichoprosopon magnum*, *T. fluviatilis*, *T. digitatum* and *T. pallidiventer*. The strain recovered from *H. spegazzinii falco* was obtained from a pool of 89 specimens collected in June of the same year at Cerro Azul. Neither agent corresponded immunologically to yellow fever or St. Louis encephalitis, other group B viruses known to occur locally. Both corresponded very closely to Ilhéus, as may be noted in the results of neutralization and cross-immunity experiments summarized in Table 1. The strain from *H. spegazzinii falco* produced illness in three of seven 2-day-old mice, 8 and 9 days, respectively, after inoculation by the intracerebral route. On third passage all infant mice inoculated intracerebrally sickened or died within 5 days and adults, within 7. Reisolation was effected after 24 days of storage of the original mosquito suspension in CO_2 ice.

The strain of Ilhéus virus recovered from *Trichoprosopon spp.* proved of especial interest because of its slow neuroadaptation. Only 2 of 8 infant mice injected intracerebrally with the original mosquito suspension developed symp-

* This investigation was supported in part by research grant E-1941 from the National Institutes of Health, U. S. Public Health Service.

TABLE 1

Results of cross-challenge and neutralization tests with isolates of Ilhéus virus

Virus strain	Logs virus neutralized by antisera against		
	Honduran	*Haemagogus*	*Trichoprosopon*
Honduran	4.0	2.5	3.3
Haemagogus	3.7	2.9	3.2
Trichoprosopon	4.6	3.0	3.5
Challenging strain	**Survival rates of challenged mice previously immunized to**		
	Honduran	*Haemagogus*	*Trichoprosopon*
Honduran	20/20*	17/19	17/18
Haemagogus	15/16	19/20	18/18
Trichoprosopon	19/20	19/19	20/20

* First number indicates the number of mice surviving challenge; the second, the number of mice challenged.

toms, which appeared on the 10th and 15th days, respectively, after inoculation. However, all 6 survivors were found immune to the later passage of more virulent material. Capacity to produce death in all adult mice injected was not achieved until the 10th consecutive passage. Second passage brain tissue showed an LD_{50} of $10^{-2.6}$ and an ID_{50} of $10^{-6.0}$; 14th passage tissue, an LD_{50} of $10^{-5.6}$ and an ID_{50} of $10^{-6.5}$. Thus increased virulence was not associated with great increase in virus titer but rather with gradual acquisition of the capacity to produce fatal central nervous system disease. Reisolation could not be attempted because of insufficiency of material. Both the isolate recovered from *H. spegazzinii* and that from *Trichoprosopon* protected immunized mice from cross-challenge with the other strain and each was neutralized by antisera against the other strain in similar titer.

DISCUSSION

The isolation of Ilhéus virus in Panama is important because it confirms the existence of this virus there. It seems unlikely, however, that the arboreal mosquitoes from which this virus was recovered play a major role in the epidemiology of the disease. Antibodies against Ilhéus virus have been found to be prevalent in the blood of rural residents of the Paya section of Darién,[4] but only one virus isolation was made from mosquitoes. Undoubtedly, recovery of the virus would have been more frequent if ground catches had been attempted. Previous isolations of Ilhéus virus have been made principally from mosquitoes of the genus *Psorophora*.[1, 5, 6] *Psorophora ferox* as well as *Aedes aegypti* and *A. serratus* also have been shown capable of transmitting the infection experimentally by bite.[5] It is of interest to note that St. Louis encephalitis virus was simultaneously active in this area, being recovered repeatedly from canopy mosquitoes and twice from human blood.[3]

SUMMARY

The first isolations of the virus of Ilhéus encephalitis in Panama are reported. The virus was recovered once from *Haemagogus spegazzinii falco* and once from mixed species of the genus *Trichoprosopon*.

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BIRDS AS HOSTS OF ILHÉUS ENCEPHALITIS VIRUS IN PANAMA*

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The importance of birds in the ecology of a number of arthropod-borne viruses is being recognized with increasing frequency. In the present publication, investigation of these vertebrates as hosts of the virus of Ilhéus encephalitis is reported. In the course of studies on the local arbor virus population here, blood specimens were collected from a wide variety of birds both in the Province of Bocas del Toro on the Costa Rican border of Panama and in the Province of Darién at the opposite end of the Republic. These blood specimens were used for possible virus isolation and also for serological studies. Ilhéus virus was recovered from sera of two of the birds, a little blue heron, *Florida caerulea*, and a keel-billed toucan, *Rhamphastos sulfuratus*, respectively. Antibodies against Ilhéus virus also were encountered in several avian sera.

MATERIALS AND METHODS

Birds were obtained both by shooting and trapping and were bled by cardiac puncture. The blood was stored in ice and forwarded by plane to the laboratory in Panama City at weekly or biweekly intervals. Most of the material arrived in satisfactory condition. Badly hemolyzed or contaminated specimens were discarded. For virus isolations the sera were diluted with equal quantities of rabbit serum saline containing 1,000 units of penicillin and 1,000 μg of streptomycin per cmm and injected intracerebrally into 2-day-old suckling mice. The viral isolates were identified by immunological comparison with a strain of the virus of Ilhéus encephalitis recovered from mosquitoes in Honduras.[1] Reciprocal cross-neutralization tests were performed with mouse hyperimmune sera prepared against the known and new strains. Also, cross-challenge experiments were conducted in which mice immunized against the homologous strain were challenged by the intracerebral introduction of 1,000 to 10,000 LD_{50} of the heterologous strains.

* This investigation was supported by research grant E-2984 from the National Institutes of Health, U. S. Public Health Service.

RESULTS

Altogether 48 sera from birds captured in Darién and 118 sera from birds captured in Bocas del Toro were injected into suckling mice for possible virus isolation. More than 40 different avian species were included, some of which have not yet been completely identified. Two recoveries of the virus of Ilhéus encephalitis were made, one from a little blue heron and one from a toucan, both captured in mid-March of 1960 in a lowland swampy section of the study area located in Bocas del Toro.

No other virus was obtained from birds in Bocas del Toro but two strains of an as yet unidentified virus were isolated in Darién. On primary isolation the heron strain (BT 871) produced illness in two of seven suckling mice on the 7th day after inoculation. By the fourth passage it produced nervous symptoms and death within 5 to 7 days in all infant and adult mice injected by the intracerebral route with 10% virus brain suspensions. The virus was reisolated from this blood after storage for 15 days in CO_2 ice. On primary isolation the viral agent recovered from toucan blood (BT 868) produced death in 1 of 7 suckling mice 13 days after inoculation. This strain also was readily adapted to adult mice, producing coma and death within 6 days of injection on the third successive passage. Reisolation was attempted but was unsuccessful. The two avian strains showed a very close cross-relationship with the type Ilhéus strain as may be noted in Table 1. There was marked immunological difference between the avian strains and St. Louis and yellow fever viruses, other group B viruses known to occur locally.

Neutralization tests also were performed with the Honduran strain of the virus of Ilhéus encephalitis and the blood of 130 birds, 62 from Bocas del Toro (captured in February and March of 1960) and 68 from Darién (captured in February, March and April of 1959).

Seven specimens neutralized 2 or more logs of the virus. These were as follows: 1 of 5 little

TABLE 1

Results of immunological tests with strains of Ilhéus virus

Serum against	Neutralization tests Virus strains, logs neutralized		
	Honduran	Heron (871)	Toucan (868)
Honduran strain	2.8	3.3	2.5
Heron (871) strain	2.8	3.7	2.7
Toucan (868) strain	2.4	3.0	3.0
Mice immune to	**Cross-neutralization tests Challenged with virus strains**		
	Honduran	Heron (871)	Toucan (868)
Honduran strain	—	12/12*	11/12
Heron (871) strain	9/10	—	10/10
Toucan (868) strain	9/10	9/9	—

* The first figure refers to the number of mice surviving challenge with the heterologous strains; the second, the number of previously immunized mice challenged.

blue herons (*Florida caerulea*) from Bocas; 1 of 3 snowy egrets (*Leucophoyx thula*) from Bocas; 1 of 3 oropendulas (*Zarhynchus wagleri*) from Bocas; 1 each of the tropical kingbird (*Tyrannus melancholicus*) and the yellow-billed cacique (*Amblycercus holosericeus*) from Bocas; 1 of 15 chickens from Darién; and 1 of 4 keel-billed toucans (*Rhamphastos sulfuratus*) from Darién.

Ilhéus virus was known to be present in mosquitoes in Paya, Darién, shortly prior to the capture of the birds.[2]

DISCUSSION

The isolations of Ilhéus virus here reported are the first to be made from birds in Central America. Further studies are needed to determine the relative importance of the little blue heron and toucan as well as other birds in the ecology of this infection in Panama. It is of interest in this connection that another group B virus, Japanese B, was repeatedly isolated from the blood of members of the family Ardeidae in Japan and antibodies to this virus were found in their blood.[3, 4] The family Ardeidae includes egrets and herons. Kissling and co-workers[5] found antibodies to equine encephalomyelitis in the heron; and in a closely related species, the white ibis, they found antibodies not only to equine encephalomyelitis but also to St. Louis. Antibodies to St. Louis virus likewise occur in the blood of toucans, herons and other birds in Panama.[6]

SUMMARY

The virus of Ilhéus encephalitis was isolated from the blood of two birds captured in March, 1960, in the Province of Bocas del Toro in Panama. Five of 62 birds obtained in Bocas and 2 of 68 birds obtained in Darién had neutralizing antibodies against this virus in their blood.

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VIRUS ISOLATIONS FROM FOREST MOSQUITOES IN SAN VICENTE DE CHUCURÍ, COLOMBIA

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In a previous paper the results of a survey of arbor virus antibodies in San Vicente de Chucurí, Colombia, a "municipio" (county) situated partly in the foothills of the Andes and partly in the valley bottom of the Magdalena River, were reported. In this municipio, jungle yellow fever (JYF) has been repeatedly active—81 human cases diagnosed between 1936 and 1959—and Venezuelan equine encephalitis (VEE) virus has been isolated twice from human beings. Furthermore, intense vaccination against yellow fever had been done there. The survey showed, among other things, high immunity rates for yellow fever, judging by neutralization tests, and varying prevalences of antibodies for Ilhéus virus and VEE in the two distinct topographical areas in San Vicente, the prevalence being higher in the valley bottom.

Virus isolation attempts from mosquitoes collected in this area from November, 1958, to March, 1960, are presented in this report, which is the second phase of a long-term study of the epidemiology of JYF and other arbor viruses in the area.

MATERIALS AND METHODS

San Vicente has been described previously.[1] Six mosquito collecting stations were established there, four (Peroles, Tierrabuena, Albania and Esterlina) in the valley bottom, at altitudes ranging from 180 to 310 meters, and two in the foothills (Trianon at 580 meters of altitude and Guadual at 800 meters). Peroles and Tierrabuena are located in tropical rain forest which is typical of that area along the middle reaches of the Magdalena River. Albania and Esterlina are located in patches of primitive forest surrounded more or less completely by clearings made for agricultural purposes. Trianon is located in a small patch of primitive forest and Guadual, in a coffee grove. In all six stations platforms were built in trees at altitudes of 13 to 26 meters above the ground.

Mosquitoes were collected once a month by two men working for 1 week, each monthly collection comprising two collections at each of the six stations. The daytime collections were made from 9 a.m. to 3 p.m. by one man working alone at each station, half the time on the elevated platform and half the time on the ground, using himself as bait. In addition captures from 6 p.m. to 7 p.m. were made with a modified "Shannon trap," using a light instead of an animal as bait. The trap described by Shannon[2] is large enough to cover a horse and is made of cloth and cloth netting. For use as a light trap, a gasoline vapor lamp is placed inside the net, towards one end, in order to give a gradient of light. The trap is then adjusted so that the sides of the net reach the ground and prevent the mosquitoes from reaching the lamp. They are picked off the outside of the net with a sucking tube.

All mosquitoes were collected with a simple mouth-suction aspirator tube, care being taken to prevent them from obtaining blood from the collector, who had yellow fever antibody in his blood plus an unknown number of other arbor virus antibodies. As soon as they were captured the mosquitoes were blown into glass jars of 1-pint size or smaller, lined with plaster of Paris and closed with a removable, inverted transparent plastic cone. In the tip of the cone was a circular opening, 7 mm in diameter, through which the mosquitoes were blown. When the collecting was finished, the jar was sealed with a screw-cap cover and placed in a thermos with ice for shipment. The next day the jars, still under refrigeration in thermos jugs, were shipped by air to the Instituto Carlos Finlay in Bogotá, where the mosquitoes were identified, classified and placed in glass tubes, flame-sealed and stored at −50°C until inoculation. The pools of mosquitoes were ground with

* A dependency of the Ministry of Public Health of Colombia which also receives financial support and technical advice from the Pan American Health Organization, Regional Office of the World Health Organization.

Alundum® in a small amount of chilled saline, 0.75% bovalbumin solution to which antibiotics were added; and, after centrifugation, the supernatant fluids were inoculated intracerebrally into a litter of 5 to 7 Swiss mice, 2 to 3 days old. The majority of the pools comprised 50 to 100 mosquitoes, which were ground in 2 to 3 ml of the diluent. For pools containing fewer mosquitoes, the diluent ranged from 0.5 to 2.0 ml, approximately proportional to the number of insects. For pools of 101 to 200, 201 to 350 and 351 to 500 mosquitoes, 2.5, 3, and 4 ml of diluent, respectively, were used. Pools of *Psorophora*, *Aedes scapularis* and *Aedes serratus*, comprising 300 or more, were ground in twice the stated amount.

The remaining supernatant fluid was sealed in glass and stored at −50°C for possible future use, *i.e.*, to confirm a virus isolation. Inoculated mice were observed for 21 days. In case a virus was isolated it was identified by means of neutralization tests with known immune sera, and, if necessary, by serological tests using known viruses and immune sera for the isolate, prepared in guinea pigs, rhesus monkeys and mice. The neutralization tests were performed by mixing 10-fold dilutions of virus with an equal volume of undiluted immune serum. The virus was titrated by mixing the dilutions of virus with an equal volume of normal guinea pig serum. The serum-virus mixtures were incubated at 37°C for 1 hour, after which they were inoculated into groups of six mice. The route of inoculation and the age of the mice were selected according to the virus used. Additional complement fixation (CF) and hemagglutination-inhibition (HI) tests were performed when indicated. For HI tests, the procedure described by Clarke and Casals,[3] using lucite plates, was followed. For CF tests the box, or checkerboard, titration system was used in which each serum was tested in increasing 2-fold dilutions beginning at 1:10, with antigen in increasing 2-fold dilutions beginning at 1:2. Acetone-ether extracted antigens were used in both tests. No isolation was considered valid unless the same virus was reisolated from the original supernatant fluid.

RESULTS

During the 17 months of the study, a total of 41,564 mosquitoes, in 748 pools, was inoculated into mice as shown in Table 1. From 12 pools a virus was isolated. Eleven of the isolates have been completely identified: 4 as VEE, 4 as Mayaro and 3 as Ilhéus. The remaining one has been classified as a virus belonging to Casals' group B by means of HI tests. No isolations of yellow fever virus have been made.

The strains of VEE, 41309, 41314, 41834 and 42170, were identified by submitting the isolates to subcutaneous neutralization tests in adult mice with a known rabbit VEE immune serum. This serum showed neutralization indices of log 5.3 with a known strain (the Kubes-Soriano strain) of equine origin, and at least log 3.2 with the four isolates from mosquitoes. Furthermore the serum of a rabbit immunized with strain 41309 showed a neutralization index of at least log 5.0 with both strain 41309 and the Kubes-Soriano strain.

The strains of Mayaro, 41097-1, 41097-2, 41097-6 and 41097-9, were identified by means of neutralization tests; the results are presented in Table 2. Three of the strains were neutralized by a Mayaro immune serum prepared with a Trinidadian strain (Tr 15537) isolated from mosquitoes. The fourth strain, 41097-2, was not tested with the Mayaro immune serum but was neutralized by the serum of a rhesus monkey immunized with strain 41097-1. The same monkey serum, as well as the serum of mice immunized with strain 41097-1, exhibited neutralizing antibodies for the Trinidadian strain of Mayaro. It must be admitted that in general the titers of the normal serum-virus mixtures were rather low. However, the data, taken as a whole, indicate that the agents were sufficiently similar to be considered as strains of the same virus. Furthermore, in HI tests performed in The Rockefeller Foundation Virus Laboratories, New York, the serum of mice immunized with strain 41097-1 had a titer of 1:320 with Mayaro antigen and gave negative results with yellow fever, Ilhéus, dengue 1, dengue 2, St. Louis, Bussuquara, Bunyamwera and VEE antigens. This same serum, which in CF tests had a titer of 1:20 with Mayaro antigen diluted 1:4, gave negative results in CF tests performed in the Trinidad Regional Virus Laboratory with the following viruses: Bunyamwera group (Kairi, Cache Valley-strain Tr 20659 and Tr 8349), Guamá group (Bimiti), California group (Melao), Simbi group (Oropouche) and ungrouped (Manzanilla, Tr 10076, Tr 11573, Tr 18462, Tr 7994, Tr 8762, Tr 9223 and Tr 20662). Strain 41097-1 kills baby mice when inoculated by the intracerebral or sub-

cutaneous route, but does not kill adult mice inoculated by either route. We have tried to adapt the strain to adult mice but so far have only adapted it to mice less than 20 days old.

TABLE 1

Summary of mosquitoes and pools tested, and pools found positive

Species and no. of mosquitoes inoculated		No. pools inoculated	
		Total	Positive
Haemagogus spegazzinii falco	107		
Haemagogus equinus	201		
Haemagogus lucifer	91		
Haemagogus spp.	45		
Total *Haemagogus*	444	57	0
Aedes leucocelaenus	2		
Aedes leucotaeniatus	1		
Aedes serratus	4,608		
Aedes scapularis	463		
Aedes fulvus	910		
Aedes angustivittatus	114		
Aedes crinifer	831		
Aedes (Ochlerotatus) spp.	7,587		
Aedes (Howardina) septemstriatus	7		
Aedes (Howardina) sp.	1		
Total *Aedes*	14,524	197	0
Psorophora ferox	9,407		
Psorophora albipes	10,411		
Psorophora cingulata	42		
Psorophora (Janthinosoma) spp.	251		
Total *Psorophora*	20,111	276	9
Mansonia venezuelensis	472		
Mansonia albicosta	387		
Mansonia (Mansonia) spp.	29		
Mansonia (Rhynchotaenia) spp.	1,330		
Total *Mansonia*	2,218	36	0
Culex spp.	2,420	60	3
Limatus durhami	227		
Limatus asulleptus	9		
Total *Limatus*	236	15	0
Sabethes chloropterus	120		
Sabethes belisarioi	33		
Sabethes quasicyaneus	15		
Sabethes undosus	105		
Sabethes spp.	2		
Total *Sabethes*	275	30	0
Trichoprosopon digitatum	35		
Trichoprosopon longipes	332		
Trichoprosopon spp.	292		
Total *Trichoprosopon*	659	34	0
Wyeomyia (Dendromyia) spp.	120		
Wyeomyia (Wyeomyia) spp.	22		
Wyeomyia spp.	180		
Total *Wyeomyia*	322	29	0
Anopheles (Nyssorhynchus) oswaldoi	159		
Anopheles (Nyssorhynchus) spp.	31		
Anopheles (Arribalzagia) punctimacula	109		
Anopheles (A.) spp.	56		
Total *Anopheles*	355	14	0
Grand total	41,564	748	12

TABLE 1—*Concluded*

The strains of Ilhéus virus, 41660, 41982 and 41986, were identified by means of the neutralization tests summarized in Table 3. First we established the identity between strains 41660 and 41982. Later we found that immune sera for Ilhéus (strain Laemmert) neutralized strain 41660 and immune sera for strains 41660 and 41982 neutralized Ilhéus virus (strain Laemmert). Finally, strain 41986 was found to be neutralized by Ilhéus immune serum. The results, as a whole, indicate that the agents are strains of the same virus. It might be of some interest to note, however, that the sera immune for strains 41660 and 41982 exhibit a neutralizing capacity for the homologous strains at least 100 times greater than that for the heterologous Laemmert strain. Nevertheless, in CF and HI tests, sera immune for strain 41660 and for strain Laemmert behave

TABLE 2

Neutralization indices of Mayaro immune sera (NI expressed as the log of LD_{50} neutralized) in intracerebral tests in 21-day-old mice with Mayaro virus, strain Tr 15537, and in 3- to 5-day-old mice with 41097 strains

Sera from	Mayaro Tr 15537	Strain 41097-1	Strain 41097-2	Strain 41097-6	Strain 41097-9
Guinea pig immunized with Mayaro strain Tr 15537	≥4.5	≥2.1	—	≥3.9	≥3.3
Rhesus monkey immunized with strain 41097-1	1.4	≥2.6	≥2.0	—	—
Mice immunized with strain 41097-1	2.1	—	—	—	—

TABLE 3

Neutralization indices of mouse immune sera prepared against three new virus isolates (NI expressed as the log of LD_{50} neutralized in intracerebral tests in adult mice)

Sera from mice immunized with	Control Ilhéus (Laemmert)	New virus isolates		
		Strain 41660	Strain 41982	Strain 41986
Ilhéus, strain Laemmert*	3.3	≥3.8	—	—
Ilhéus, strain Laemmert*	4.6	3.3	—	4.6
Strain 41660	3.1	≥5.3	≥5.3	—
Strain 41982	3.2	≥5.3	≥5.4	—

* Sera from different groups of mice.

in exactly the same way with antigens prepared either with strain 41660 or with the Laemmert strain.

The unidentified virus 41922 yields antigens which agglutinate goose red cells and has been found to belong to Casals' B group. The optimum reactions are observed at pH 6.6 and at a temperature of 20°C.

Sera immune to St. Louis, Ilhéus, dengue 1 and dengue 2 produce the HI phenomenon with the antigens prepared with virus 41922, but usually they exhibit higher titers with their homologous antigens. Immune mouse sera for virus 41922 produce inhibition of the agglutinating capacity of the homologous antigen as well as of the antigens prepared with Ilhéus, dengue 1, dengue 2, St. Louis and yellow fever (17D). However, when the 41922 immune sera are absorbed with St. Louis virus, with 7.2 grams of infected mouse brain per 1 ml of serum according to Clarke's method,[4] all the heterologous antibodies are removed, leaving only antibodies for virus 41922. This fact agrees with the results of the neutralization tests, which show that virus 41922 is not neutralized by sera immune to the five aforementioned B agents nor are these viruses neutralized by immune sera prepared against our isolate. The complement fixation tests (box titrations) made with several group B antigens, which are summarized in Table 4, also indicate that virus 41922 belongs to group B, but is different from St. Louis, Ilhéus and dengue 1.

The four strains of Mayaro were isolated from four pools of 50 mosquitoes each, taken from a group of 447 specimens of *Psorophora* composed of 119 *P. albipes*, 154 *P. ferox* and 174 either *ferox* or *albipes* collected on the valley bottom stations on November 10–12, 1958.

Two of the strains of VEE, 41309 and 41314, were isolated from two pools of 50 mosquitoes each, taken at random from a group of 969 *Psorophora* composed of 932 *P. albipes* and 37 *P. ferox* collected in Peroles and Tierrabuena on November 24, 1958. The two remaining strains of VEE virus were isolated from two pools of *Culex spp.* collected in the light trap in Tierrabuena: one, 41834, on May 7, 1959; and the other, 42170, on September 15, 1959.

The strains of Ilhéus were isolated from pools of *P. ferox*: one (41660) composed of 48 specimens collected in Tierrabuena on January 28, 1959; one, 41982, composed of 443 specimens collected in Albania on July 7, 1959; and one, 41986, composed of 344 collected in Esterlina on July 7, 1959.

The B agent, 41922 not yet identified, was isolated from a pool of 95 *Culex spp.* collected in Tierrabuena on June 30, 1959, during the evening.

DISCUSSION

All the virus isolations were made from mosquitoes collected in the valley bottom stations, mainly in those situated in the rain forest of the Magdalena River. This fact may be related to the relative abundance of mosquitoes there. Of all the mosquitoes inoculated, which

TABLE 4

Complement fixation tests (box titrations) made with St. Louis, Ilhéus, dengue 1, and virus 41922 antigens

Immune serum	Antigen			
	41922	St. Louis	Ilhéus	Dengue 1
41922	1:80/1:32*	1:20/1:16	1:10/1:8	0/0
St. Louis	0/0	1:80/1:256		
Ilhéus	0/0	0/0	1:20/1:64	
Dengue 1	0/0		0/0	1:20/1:2

* Titer of serum/titer of antigen.

number corresponds practically to 100% of the collected specimens, 38,433 were captured in the valley bottom and only 3,131 in the foothills.

The isolation of Ilhéus and Mayaro viruses confirms what had been anticipated as a result of the serological studies in San Vicente. Neutralizing antibodies to Ilhéus virus were found in 14.6% of the valley bottom residents but in only 3.4% of the residents of the foothills. HI antibodies for Mayaro virus in high titers were found in 3 of 90 residents of the foothills; however, no residents of the valley bottom were tested for this virus.

The isolation of VEE virus from forest mosquitoes fits nicely with our previous findings: two isolations of the virus from human beings living in the valley bottom; the high average prevalence of antibodies in residents of that area (34.1%) ranging up to 71%; and 11 of 17 monkeys captured in the region showing antibodies. These findings support our belief that in San Vicente VEE virus is closely associated with tropical rain forest.

Our strains of Ilhéus virus have been isolated from *Psorophora ferox*. In Trinidad this virus also was isolated recently from *P. ferox*, *Aedes serratus*, *Aedes scapularis*, *Culex caudelli* and from mixed pools which contained *Psorophora spp.*[5, 6]

According to the literature at our disposal, this is the first time that Mayaro virus has been recovered from *Psorophora* mosquitoes captured in the forest. Besides man, the virus had been previously isolated from *Mansonia venezuelensis* in Trinidad.[7]

VEE virus has been isolated recently from five pools of forest mosquitoes in Brazil.[8] Two pools consisted of sabethine mosquitoes, one of *Haemagogus sp.*, one of *Aedes serratus*, and the fifth of *Aedes serratus*, *Aedes scapularis*, *Psorophora ferox* and one sabethine mosquito. Here we have isolated the virus from *Psorophora* and *Culex spp.*

The failure to isolate yellow fever virus must be viewed in the light of the fact that only 444 *Haemagogus* were inoculated.

SUMMARY

Twelve strains of arbor virus were isolated from 41,564 forest mosquitoes collected in San Vicente de Chucurí, Santander, Colombia, during the 17-month period from November, 1958, to March, 1960. Mosquitoes were collected with human bait and a very simple light trap and shipped under refrigeration by air to a distant laboratory where they were identified and inoculated into infant mice.

The virus isolates include four strains of Mayaro, all from *Psorophora*; four strains of Venezuelan equine encephalitis (VEE), two from *Psorophora* and two from *Culex*; three strains of Ilhéus, all from *Psorophora ferox*; and one strain from *Culex*, unidentified as yet but clearly belonging to Casals' group B.

All isolations were made from mosquitoes captured in the four valley bottom stations where about 92.5% of the mosquitoes were collected. Earlier studies in San Vicente had shown much higher prevalence rates of antibody to Ilhéus and to VEE in valley bottom residents than in foothill residents. This may be correlated with the prevalence of mosquitoes, which are much more abundant in the valley bottom area.

ACKNOWLEDGMENTS

We want to express our sincere thanks to Drs. Max Theiler, Wilbur G. Downs and Leslie Spence for their help in the serological work, to Dr. J. Austin Kerr for his advice, and to Dr. Augusto Gast Galvis, Director of the Carlos

Finlay Institute, for his unfailing assistance in all matters scientific and administrative.

ADDENDUM

Dr. Max Theiler, who received the group B virus 41922, has recently informed us that CF and HI studies carried out in The Rockefeller Foundation Virus Laboratories by Dr. Oscar de Souza Lopes indicate that this virus is closely related, possibly identical with, Bussuquara.

Ten more virus strains were isolated from mosquitoes collected in San Vicente during the last 9 months of 1960. One strain, isolated from a pool of *Culex spp.*, has been found to be identical with the group B virus 41922. Six (five from pools of *Psorophora ferox* and one from a pool of *Aedes serratus*) have been identified as Mayaro. The remaining three strains have not been studied yet.

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THE VIRULENCE OF WEST NILE VIRUS AND TP 21 VIRUS AND THEIR APPLICATION TO A GROUP B ARBOR VIRUS VACCINE*

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The possibility of using the serologic relationships that exist between members of the group B arbor viruses to obtain a vaccine against the group B viruses has been discussed by a number of workers.[1-8] Because of the questionable efficacy of the formalin-inactivated vaccines and the large number of viruses capable of causing clinical disease in the group B arbor viruses, it is doubtful whether homotypic vaccines would be the most effective method of immunizing against these diseases.[9, 10] Studies in this laboratory over the past 6 years have been concerned with the possibility of obtaining a triple vaccination procedure effective against the group B arthropod-borne viruses.[1, 2]

Early investigations indicated that an attenuated strain of West Nile virus might be of value in the development of such a vaccine for man.[1-4] This virus was chosen on the following basis. The results of Casals[11] indicate that with regard to both immunological relationships and clinical disease the group B viruses may be divided into three main sub-groups: (a) the yellow fever-dengue groups, (b) the Japanese B encephalitis-St. Louis encephalitis-Murray Valley encephalitis-West Nile group, and (c) the Russian spring-summer complex of viruses. In determining the choice of viruses for the vaccine, the live virus under investigation was inoculated into chickens or monkeys, and this was followed approximately 1 month later by an injection of formalin-inactivated virus of one of the other sub-groups.

Such experiments showed, for example, that if Theiler's 17D-yellow fever vaccine was given, and this followed by an injection of formalin-inactivated Japanese B virus vaccine, there was no detectable appearance of neutralizing antibodies to Japanese B virus. Similar results were found when live dengue virus was substituted for yellow fever virus.[2] When animals were given West Nile virus instead of yellow fever virus and subsequently inoculated with formalin-inactivated Japanese B virus vaccine, they produced Japanese B virus neutralizing antibodies.[2] Imam and Hammon made similar observations with this latter system.[4] Formalin-inactivated West Nile virus vaccine, even when concentrated 30-fold, did not behave as living West Nile virus.[12]

An additional reason for choosing West Nile virus was that in nature this virus has been found to be associated in the vast majority of the cases with a relatively mild disease in human beings in comparison to the group B viruses associated with such diseases as Japanese B encephalitis.[13, 14] Since an inactivated virus could not be used as discussed above, this initially relatively low virulence of West Nile virus for man made the attenuation problem safer.

If primates were given an injection of living 17D-yellow fever vaccine, followed by an injection of living West Nile virus, followed by a third injection of formalin-inactivated Russian spring-summer virus vaccine, protection was obtained against viruses of group B mentioned above[12] which cause significant clinical illness in man. Protection was determined on the basis of a marked lowering and shorter duration of viremia when the animal was challenged with virulent virus. It was essential that one member of each of the three sub-groups of the group B arbor viruses be included in the vaccination procedure.[12] Preliminary studies in primates in this laboratory indicate that the ideal vaccination procedure for man would consist of 17D-yellow fever vaccine, living Japanese B virus, and living Russian spring-summer virus in sequence.[12] Of the combinations tried, this combination gave neutralizing antibody titers of the highest levels and longest duration against members of the group B arbor viruses that are of clinical importance for man. However, neither an attenuated Japanese

* This work was supported in part by a contract from the Department of the Army, Office of The Surgeon General, Washington, D. C., on recommendation of the Commission of Viral Infections of the Armed Forces Epidemiological Board. The work was also supported by a grant from The Rockefeller Foundation.

B virus isolate nor a Russian spring-summer virus isolate which could be used for vaccination of human beings is available at this time.

It should be emphasized that the available data suggest that such a triple vaccination might reduce the frequency and severity of clinical disease but might not completely prevent all symptoms associated with these virus diseases. This conclusion is based on the observation that the vaccine does not completely prevent the viremia associated with many of these virus infections in the experimental animals that were used, but markedly reduces the height and duration of viremia. Obviously, field trials in man will need to be carried out before definite conclusions can be drawn.

Since the West Nile virus plays such a necessary role in the suggested vaccination procedure against the group B virus infections in man, it was important to undertake a comprehensive study of the virulence properties of this agent.

In previous work, the characterization of three strains of West Nile virus in terms of virulence and immunogenic potential for laboratory animals and man has provided a basis of comparison for the study of other strains of West Nile virus.[15] These three strains were found to be distinctly graded in terms of their virulence and immunogenic potential for both man and laboratory animals.[15]

A recent epidemic of West Nile infection which occurred in Israel[11] has made available additional strains of West Nile virus which have not been passed extensively in laboratory animals. These virus isolations were made from human beings in whom the disease was well studied clinically. Serological studies performed with convalescent-phase sera of persons infected during the epidemic showed cross-neutralizing antibodies against related Japanese B encephalitis (Jap-B) virus and St. Louis encephalitis virus in addition to the homologous West Nile virus.[16]

It is the purpose of this paper to compare the virulence of one of these Israel isolates to the three reference strains mentioned above. It will be shown that this Israel strain of West Nile virus after being passed in chick embryo tissue cultures has biological properties which may make it suitable for preliminary studies in man.

Details are also presented of the efficacy of the triple vaccination procedure when vaccinated primates are challenged with various virulent group B viruses.

MATERIALS AND METHODS

All procedures except the method of passage of virus in chick embryo tissue cultures have been described previously.[15] The chick embryo tissue cultures and plaque methods were carried out as described by Buckley.[17] The chick embryos were left at 6°C for 24 hours prior to use. All viruses unless otherwise noted were described previously.[15] The TL-443 isolate of West Nile virus was obtained as the fourth mouse brain passage from Dr. N. Goldblum of Israel. This virus was isolated during an epidemic of West Nile infection.[14] The hemagglutination experiments were carried out according to the method of Clarke and Casals.[18] The two strains of Russian spring-summer virus and the dengue viruses were obtained from the Walter Reed Army Institute of Medical Research and the Virus Laboratories of The Rockefeller Foundation of New York. The Wesselsbron virus was obtained from Dr. Max Theiler and was in the 13th mouse brain passage.

The spider monkeys utilized in the tests described in this paper were obtained from Ecuador and Colombia and were bled upon receipt to check for the presence of neutralizing activity against yellow fever, Japanese B, West Nile, and dengue 1 viruses. Approximately fifty 3-day-old mouse intraperitoneal (i.p.) LD_{50} of each virus strain were utilized in the screening tests for neutralizing antibodies except in the case of dengue 1 virus where approximately 50 mouse intracerebral (i.c.) LD_{50} were used. The results of these tests showed that the sera of 77% of the 130 animals studied possessed no neutralizing activity against the group B viruses listed above. It is interesting to note that in those animals in which neutralizing activity was detected, yellow fever and dengue viruses were the viruses neutralized. The chimpanzees used in this work were also screened in the above manner for neutralizing antibodies.

After each primate was inoculated with the various specimens under study, the animal was observed daily for 30 days by the same observer. The individual who made the observations did not know what viral specimens the animals had received. The clinical observations were of the same type as those made in the earlier work.[15]

TABLE 1

Titer of Israel isolates of West Nile virus in various laboratory animals

Strain no. and mouse passage (M) no.	3-day-old mice		8-10 gram mice		6-week-old hamsters i.p.†
	i.c.*	i.p.†	i.c.*	i.p.†	
TL-2-M8	8.0 ± 0.31‡	8.3 ± 0.24	7.5 ± 0.31	5.0	3.0 ± 0.21
TL-428-M4	7.4 ± 0.29	7.4 ± 0.21	6.3 ± 0.24	5.0	2.7 ± 0.24
TL-443-M4	7.4 ± 0.30	7.3 ± 0.41	6.7 ± 0.14	5.0	3.0 ± 0.40
TL-450-M5	8.0 ± 0.21	8.2 ± 0.31	7.5 ± 0.34	5.0	5.3 ± 0.29
PH-241-M7	7.3 ± 0.21	7.0 ± 0.19	6.4 ± 0.18	5.0	
PH-541-M5	8.0 ± 0.24	8.0 ± 0.24	7.3 ± 0.29	5.0	

* i.c.—intracerebral.

† i.p.—intraperitoneal.

‡ Numbers represent the reciprocal of the logarithm (base 10) of the titration endpoints. All titrations were repeated 3 times except the intraperitoneal inoculation of 8- to 10-gram mice which was carried out once. All titers are adjusted to an inoculum volume of 0.03 ml.

The clinical manifestations of encephalitis noted were those listed in the footnote to Table 18.

All rhesus monkeys used in the intracerebral tests were free of detectable neutralizing antibodies against West Nile virus as determined by the method described above. They also showed no hemagglutination inhibiting antibodies when the West Nile virus was used as antigen.

In all the tables, when titration figures are given, they represent the reciprocal of the logarithm (base 10) of the titrations.

RESULTS

Selection of isolates of West Nile virus from Israel. Several isolates recovered during the epidemic in Israel have been made available to this laboratory by Dr. N. Goldblum. The isolates were repassed in suckling mice as 10% suspensions of the reconstituted lyophilized virus received. The brains were then harvested as 10% suckling mouse brain suspensions diluted in 10% normal rabbit serum in physiological saline (10% NRS). These preparations were titrated intracerebrally in 3-day-old mice and 8- to 10-gram mice, and intraperitoneally in 3-day-old mice and 8- to 10-gram mice and hamsters (Table 1). Virus was also inoculated subcutaneously into 4-day-old White Rock chickens and circulating virus was titrated at 24-hour intervals between 24 and 120 hours after the initial virus inoculation (Table 2). As a result of these tests, two of the isolates, designated TL-443 and TL-450, were selected for further study. Isolate TL-443 was chosen because it did not kill chickens. The other isolate was selected at random. Inoculation of rhesus monkeys intracerebrally with 1,000 8- to 10-gram mouse i.c. LD_{50} of one or the other of these two virus isolates indicated the TL-450 to be the more encephalitogenic. Since preliminary studies showed the TL-443 isolate on intracerebral inoculations in rhesus monkeys to be less encephalitogenic than the Egypt 101 strain studied previously, further characterization was continued with only the TL-443 isolate.

Comparative studies of the ability of the Egypt 101 and TL-443 isolates of West Nile virus to produce viremia in chickens and spider monkeys and to produce encephalitis in rhesus monkeys. Since the results with the human reference strains described in the previous paper[15] indicated the Egypt 101 strain to be the most virulent, the virulence of the TL-443 virus was compared to that of the Egypt 101 strain by injecting subcutaneously comparable inocula of either virus strain into White Rock chickens and spider monkeys.

The results with both strains were practically indistinguishable. As indicated in Tables 3 and 4, chickens or spider monkeys inoculated subcutaneously with either TL-443 or the 101 strain developed viremia which lasted at least 5 to 6 days. The maximum titers in chickens were about 1,000 8- to 10-gram mouse i.c. LD_{50} of circulating virus per 0.03 ml of blood between 48 and 96 hours after inoculation.

When inoculated intracerebrally into rhesus monkeys, TL-443 was somewhat less encephalitogenic than the 101 strain (Table 5).

TABLE 2

Viremia induced in 4-day-old White Rock strain chickens following subcutaneous inoculation of indicated dose of West Nile virus, Israel isolates

Strain and quantity of virus inoculated	Chicken no.	Fatalities in groups of 5 mice inoculated with 20% blood at given time interval*			
		24 hr	48 hr	72 hr	96 hr
TL-2-M7, 1,000 8–10 gram mouse i.c. LD_{50}	133	5	5	5	5
	132	5	5	5	5
	131	3	5	5	—†
	130	5	5	5	5
	129	1	5	5	5
TL-428-M4, 200 8–10 gram mouse i.c. LD_{50}	138	5	5	5	3
	137	3	5	5	—†
	136	2	5	5	5
	135	5	5	5	5
	134	5	5	5	—†
TL-443-M4, 200 8–10 gram mouse i.c. LD_{50}	143	5	5	5	5
	142	4	5	5	1
	141	2	5	5	5
	140	0	5	5	5
	139	5	5	5	5
TL-450-M5, 20 8–10 gram mouse i.c. LD_{50}	148	5	5	—†	—
	147	0	5	5	1
	146	5	5	5	5
	145	5	5	5	5
	144	5	5	5	—†
PH-241-M7, 200 8–10 gram mouse i.c. LD_{50}	128	5	5	5	—†
	127	4	5	5	5
	126	2	5	5	5
	125	5	5	5	5
	124	5	5	5	5
PH-541-M5, 1,000 8–10 gram mouse i.c. LD_{50}	123	5	5	5	5
	122	5	5	5	5
	121	5	5	5	5
	120	5	5	5	5
	119	5	5	—†	—

* These represent specific deaths.
† Chicks died.

Immunologic studies of Egypt 101 and TL-443 West Nile isolates. The sera of White Rock chickens and spider monkeys inoculated with TL-443 virus were tested at several intervals after infection to determine the homologous neutralizing antibody titer against West Nile virus as well as the titer against certain heterologous but related group B viruses. The neutralizing indices obtained with this isolate were compared to those of the Egypt 101 strain which was studied under similar conditions. The indices calculated are shown in Table 6, which presents the data obtained from the sera of the chickens infected when 4 days old as well as from the sera of 2- to 4-pound spider monkeys. The same methods as described previously were used for determining the neutralizing indices.[15]

Neutralization tests performed with the sera of mice and chickens inoculated with formalin-inactivated virus vaccines indicate that in chickens, but not in mice, the TL-443 virus may be the more immunogenic (Table 7). However, such results should be considered as preliminary data subject to further confirmation.

Challenge of spider monkeys given 17D-yellow fever vaccine with TL-443 West Nile isolate. As the vaccination procedure involves giving the West Nile virus after 17D vaccine,[2] experiments were carried out in spider monkeys to see what effect a previous inoculation of 17D would have on a subsequent infection with West Nile isolate TL-443. This virus has virulence properties different from Egypt 101 virus which had been used previously in similar experiments. Monkeys which had no detectable neutralizing antibodies to 17D virus were given either undiluted 17D virus, as prescribed for human inoculation, or an inoculum containing $\frac{1}{100}$ of the prescribed dose. The viremia induced with 17D was of a low grade and no significant difference was noted between the two groups of monkeys.

Two months after inoculation with 17D virus, each monkey was bled to obtain serum for neutralization tests and all monkeys together with uninoculated controls were challenged with 500 8- to 10-gram mouse i.c. LD_{50} TL-443 virus by the subcutaneous route. Blood was removed at 24 hour intervals and tested intracerebrally in 8- to 10-gram mice to estimate the amount of circulating virus and its duration. The results of the test indicate that 5 of the 10 monkeys previously inoculated with 17D virus were completely protected against subsequent challenge with West Nile virus, as measured by viremia studies (Table 8). In yellow fever vaccinated monkeys that showed West Nile viremia, the viremia was of shorter duration. Such protection was found to occur in spite of the uniform absence of detectable neutralization activity against West Nile virus

TABLE 3

Comparison of viremia induced in White Rock chickens infected subcutaneously with comparable doses of Egypt 101 strain or TL-443 virus

Virus strain	Age of chicken (days)	Dose of virus*	Mean titer of virus in the blood					No. infected/ total no. inoculated
			24 hr	48 hr	72 hr	96 hr	120 hr	
Egypt 101	4	200	1.5	2.7	3.5	3.0	1.7	8/8
	9	300	1.2	1.5	2.3	1.7	Trace	10/10
	14	1,000	1.5	1.7	2.3	1.3	Trace	10/12
TL-443	4	100	1.3	2.7	3.0	3.3	2.3	8/8
	9	300	1.5	2.0	1.7	1.5	Trace	10/10
	14	500	1.3	1.5	1.0	Trace	Trace	12/12

* Numbers represent 8- to 10-gram mouse i.c. LD_{50} virus inoculated into each chicken. Trace signifies a titer of about 0.7.

TABLE 4

Comparison of viremia induced by Egypt 101 strain or TL-443 upon subcutaneous inoculation in 2- to 4-pound spider monkeys (Ateles geoffroyi)

Virus strain	Dose	Monkey no.	Virus in blood*							
			48 hr		72 hr		96 hr		120 hr	144 hr
			20%	2%	20%	2%	20%	2%	20%	20%
Egypt 101	2,000 8–10 gram mouse i.c. LD_{50}	10	0	0	1	0	3	1	0	0
		11	0	0	4	0	0	0	0	0
		12	0	0	4	4	4	0	4	0
		13	4	1	4	2	3	0	1	0
		14	3	0	3	1	4	1	4	1
TL-443	3,000 8–10 gram mouse i.c. LD_{50}	15	0	0	1	0	4	3	2	0
		16	2	0	4	2	4	1	4	0
		17	1	0	4	2	4	0	4	2
		18	1	0	4	3	2	0	2	2
		19	1	0	4	2	4	2	4	1

* Number of specific deaths in groups of 4 mice inoculated intracerebrally with either 20% or 2% blood.

before challenge. These neutralization tests were carried out using the intracerebral challenge. Similarly, no antibody against yellow fever virus was detected at the same interval after infection in the sera of the monkeys inoculated with West Nile virus alone.* However, slight neutralizing activity against dengue 1 virus, usually thought more closely related to yellow fever virus, was detected in the postinfection sera of some of the monkeys in both groups (Table 9).

Table 9 shows no difference in the antibody response to the West Nile, Japanese B, and St. Louis encephalitis viruses in the monkeys that received both yellow fever and West Nile viruses as compared with the response in the monkeys that received only West Nile virus; however, the response to dengue 1 was somewhat greater in the former.

Table 10 shows that monkeys given only the TL-443 virus as well as those receiving this virus after yellow fever vaccine are resistant to

* Dr. Max Theiler has informed us that, using human sera from West Nile infections in Egypt and the intraperitoneal test, he was able to show neutralizing antibodies against yellow fever virus. The sensitivity of the intraperitoneal test for detecting neutralizing antibodies has been discussed in a previous paper[15] from this laboratory.

TABLE 5

Intracerebral pathogenicity of Egypt 101 and TL-443 virus strains in rhesus monkeys

Virus strain	Virus dose*	No. inoculated	No. showing no signs of illness	No. showing clinical manifestations of encephalitis	Deaths due to encephalitis
Egypt 101	50	6	2	4	1
	1,000	8	1	7	1
	100,000	8	0	8	4
TL-443	100	8	8	0	0
	5,000	8	5	3	1
	500,000	8	1	7	1

* Dose given in terms of 8- to 10-gram mouse i.c. LD_{50} inoculated intracerebrally into monkeys.

8- to 10-gram mouse i.c. LD_{50} of 17D vaccine. Two months later they received 3,000 8- to 10-gram mouse i.c. LD_{50} of TL-443. Three months after 17D, they received one injection of formalin-inactivated Russian spring-summer virus vaccine. All inoculations were given subcutaneously. The Russian spring-summer encephalitis (RSSE) virus vaccine was made from the Moscow strain of Far Eastern Russian spring-summer virus.[19] One month after the third inoculation, the various groups of monkeys shown in Table 11 were challenged subcutaneously with 300 8- to 10-gram mouse i.c. LD_{50} of the Moscow strain of Russian spring-summer encephalitis virus. It is clear that the triple inoculation gave some protection against this virus as determined by viremia studies. Similar

TABLE 6

Neutralization indices of sera from experimental infections with Egypt 101 and TL-443 strains of West Nile virus

Virus strains	Animals*	Antibody for indicated viruses in 6-week postinfection sera†			
		West Nile	Jap B	St. Louis	Dengue 1
Egypt 101	Chickens	3.4 ± 0.41	1.9 ± 0.21	1.2 ± 0.22	1.5 ± 0.18
	Monkeys	3.8 ± 0.28	1.7 ± 0.19	1.3 ± 0.24	1.1 ± 0.11
TL-443	Chickens	3.9 ± 0.29	1.9 ± 0.31	1.7 ± 0.16	1.9 ± 0.17
	Monkeys	3.8 ± 0.32	1.9 ± 0.32	1.6 ± 0.14	1.3 ± 0.12

* Chickens used in the experiments were infected when 4 days old. Monkeys used were *Ateles geoffroyi*.

† Data presented are the mean and standard deviation of calculated neutralizing indices. Dengue viruses 2, 3, and 4 gave results similar to those of dengue 1 virus.

challenge with Japanese B virus as indicated by viremia studies. Similar results have been obtained previously in this laboratory with chicks.[15]

*The efficacy of the triple vaccination procedure against dengue viruses, types 3 and 4, and the Russian spring-summer group of viruses.** Additional experiments were carried out to test the effect of the triple vaccination procedure against members of the Russian spring-summer group of viruses.

Spider monkeys were inoculated with 100,000 results were obtained when spider monkeys vaccinated as described above were challenged with the Omsk virus of the Russian spring-summer complex.

It should be noted that the viremia produced by the Russian spring-summer encephalitis virus was not completely eliminated in 2 of the 6 monkeys which were vaccinated by the triple inoculation procedure. However, there was more protection than that observed in the monkeys given only the one inoculation of formalin-inactivated Russian spring-summer virus vaccine. It should also be noted that one injection of formalin-inactivated RSSE vaccine did confer some protection in 3 of 6 monkeys against challenge with Russian spring-summer encephalitis virus confirming the work of Morris *et al.*[19]

Additional experiments were carried out with

* For the experiments involving Russian spring-summer encephalitis viruses and dengue viruses primates were chosen which had no detectable neutralizing antibodies against these viruses in their sera as determined by the procedures described under Methods. They also showed no hemagglutination inhibiting antibodies against Russian spring-summer encephalitis virus, Moscow strain, or dengue type 1 virus.

TABLE 7

*Neutralizing antibodies induced in young chickens and juvenile mice following intraperitoneal inoculation with formalin-killed virus**

Virus strain and titer before inactivation (per 0.03 ml)	Virus titer inoculated (total)†	Serum from	Neutralization test Sera tested against				
			50 WN‡	500 WN‡	30 JB‡	30 SL‡	30 D1‡
Egypt 101 (7.0)	$3 \times 10^{8.2}$	Pooled mouse	8	8	4	0	2
	$3 \times 10^{8.5}$	Chicken 1	4	0	0	0	0
		2	8	1	0	0	0
		3	1	0	0	0	0
		4	3	1	0	0	0
		5	4	0	0	0	0
TL-443 (6.8)	$3 \times 10^{7.6}$	Pooled mouse	8	8	4	2	3
	$3 \times 10^{7.9}$	Chicken 1	2	0	0	0	0
		2	8	5	1	0	0
		3	8	7	6	1	2
		4	8	8	6	3	3
		5	7	6	5	2	2
Controls		10% NRS	0	0	0	0	0

* 5-day-old chickens given 1.0 ml formalin-killed virus followed by second injection of 1.0 ml 10 days later. Third injection of 1.0 ml given 24 days after first injection. 8- to 10-gram mice given 0.5 ml according to same schedule. Both groups bled 38 days after first inoculation.

† Determined from infectious titer before inactivation.

‡ Number of survivors remaining from 8 suckling mice inoculated i.p. with test material except with dengue virus where intracerebral test had to be used. WN represents West Nile virus; JB—Japanese B; SL—St. Louis; D1—dengue virus strain 1 (Hawaiian).

TABLE 8

Viremia induced by subcutaneous injection of 500 8- to 10-gram mouse i.c. LD_{50} TL-443 virus in spider monkeys previously immunized with yellow fever virus and compared with yellow fever non-immune controls*

Group	Monkey no.	Amount of virus in blood†						
		48 hr		72 hr		96 hr		120 hr
		20%	2%	20%	2%	20%	2%	20%
Yellow fever immunes	1	2	0	2	0	1	0	0
	3	0	0	2	0	4	0	0
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	6	3	0	4	1	4	0	1
	8	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0
	10	1	0	4	2	3	1	0
	11	0	0	1	0	0	0	0
	13	0	0	0	0	0	0	0
Yellow fever non-immune controls	16	2	0	4	3	4	3	2
	17	2	0	4	2	4	2	4
	18	1	0	3	1	4	3	3
	20‡	2	0	3	0	2	0	0
	21	1	0	4	3	4	2	4
	22	2	0	4	2	4	1	3

* Yellow fever virus is 17D.

† Number of fatalities in groups of 4 mice inoculated intracerebrally with either 20% or 2% blood.

‡ Monkey no. 20 possessed antibody capable of neutralizing both yellow fever and dengue before challenge with West Nile virus.

TABLE 9

Neutralization indices of pre- and post-experimental sera of spider monkeys used in 17D West Nile experiment

Experimental group	Monkey no.	Pre-experimental Neutralizing index of sera				Post-17D,pre-TL-443 Neutralizing index of sera				Post-TL-443 Neutralizing index of sera†				
		WN*	JB	YF	D1	WN	JB	YF	D1	WN	JB	SL	YF	D1
A. 17D and TL-443 (monkeys given undiluted 17D virus; titer: $10^{5.7}$ in 8–10 gram mice i.c.)	1	0‡	0	0	0	0	0	3.2	0	3.5	2.3	0	3.5	1.7
	2	1.3	0	>1.7	>1.7	2.7	1.0	3.5	3.5					
	3	0	0	0	0	0	0	3.7	1.3					
	4	0	0	0	0	0	0	3.2	0	>3.7	2.7	1.3	>3.7	>2.7
	5	0	0	>1.7	0	0	0	3.5	1.3	3.7	1.7	0	>3.7	>2.7
	6	0	0	0	0	0	0	2.7	0	4.1	2.6	1.2	3.4	2.4
	7	0	0	>1.7	>1.7	0	0	3.7	2.5					
(Monkeys given 100-fold dilution 17D virus; titer: $10^{3.7}$)	8	0	0	0	0	0	0	3.7	2.0	3.5	2.7	2.0	>3.7	>2.7
	9	0	0	0	0	0	0	3.0	0					
	10	0	0	0	0	0	0	3.7	2.7	>3.7	2.2	1.9	>3.7	>2.6
	11	0	0	0	0	0	0	2.7	1.7	3.3	1.5	1.7	3.7	>2.7
	12	0	0	>1.7	0	0	0	3.7	2.3					
	13	0	0	0	0	0	0	3.0	0	>3.7	2.5	1.7	>3.7	>2.7
	14	0	0	0	0	0	0	3.2	1.3	3.6	2.1	1.8	>3.7	2.5
B. (Monkeys given 500 8–10 gram mouse i.c. LD_{50} TL-443 virus)	16					0	0	0	0	3.3	2.7	1.7	0	1.0
	17					0	0	0	0	3.0	2.7	1.0	0	0
	18					0	0	0	0	3.5	2.5	2.0	0	0.7
	19					0	0	0	0					
	20					0	0	>1.7	>1.7	>3.7	>2.7	2.3	>3.7	>2.7
	21					0	0	0	0	3.7	2.0	0	0	1.3

* WN represents West Nile virus; JB—Japanese B; SL—St. Louis; YF—yellow fever (17D); D1—dengue virus strain 1 (Hawaiian).

† See Table 10.

‡ Neutralizing index. 0 means none detected (less than 0.7).

the dengue viruses, types 3 and 4, which Hammon and his group have recently isolated. These workers have presented evidence that these dengue viruses are associated with a type of hemorrhagic disease in humans.[20] Eight chimpanzees were inoculated with the triple inoculation procedure discussed in Table 11. After 1 month, four of the chimpanzees were challenged subcutaneously with 7,200 3-day-old mouse i.c. LD_{50} of type 3 dengue virus and four of the chimpanzees with 6,200 3-day-old mouse i.c. LD_{50} of type 4 dengue virus. Table 12 shows that the vaccine gave some protection against types 3 and 4 of dengue virus as determined by these viremia studies.

The efficacy of the triple inoculation procedure against Wesselsbron virus. Recent studies in South Africa have uncovered a new virus which causes clinical disease in man and appears, on the basis of antibody studies, to be widespread in the population.[21, 22] This agent has been given the name Wesselsbron virus and is a member of the group B viruses.[22] Studies were undertaken to determine what effect the 17D-West Nile-Russian spring-summer triple inoculation procedure might have on the multiplication of the Wesselsbron virus in chimpanzees. These experiments were carried out in a way similar to those described in Table 11. From Table 13, it can be seen that the group of chimpanzees given the triple inoculation showed lower concentrations of Wesselsbron virus in the blood and a shorter duration of viremia than did the control animals.

Efficacy of the triple inoculation procedure against challenge with St. Louis encephalitis virus in spider monkeys. In view of the low neutralizing antibodies to St. Louis encephalitis virus found in the monkeys given 17D plus West Nile virus,

TABLE 10

*Viremia induced by subcutaneous inoculation of 200 8- to 10-gram mouse i.c. LD_{50} Japanese B virus in spider monkeys given combined 17D and West Nile inoculations, monkeys given West Nile only, and controls**

Group	Monkey no.	No. of fatalities in groups of 5 8–10 gram mice inoculated intracerebrally with 20% blood		
		48 hr	72 hr	96 hr
17D plus WN	1	0	0	0
	4	0	0	0
	8	0	0	0
	11	0	0	0
	13	0	0	0
WN only	16	0	0	0
	17	1	3	0
	18	0	0	0
	20	0	0	0
	21	0	0	0
Controls	22	3	5	5
	23	0	0	0
	24	5	5	5
	25	4	5	5
	26	2	5	4
	27	3	5	5

* Monkeys 1, 4, 8, 11, 13, 16, 17, 18, 20, and 21 are those shown in Table 9. The control monkeys showed no detectable neutralizing antibodies to Japanese B virus when screened in the manner described in Methods. The serum of monkey 23 neutralized 17D and dengue viruses.

TABLE 11

Viremia induced by subcutaneous inoculation of 300 8- to 10-gram mouse i.c. LD_{50} Russian spring-summer virus in spider monkeys given combined 17D-West Nile plus one injection of formalin-inactivated Russian spring-summer virus vaccine, in monkeys given 17D-West Nile virus, in monkeys given one injection of killed Russian spring-summer vaccine, and in controls

Group	Monkey no.	No. of fatalities in groups of 5 8–10 gram mice inoculated intracerebrally with 2% blood			
		48 hr	72 hr	96 hr	120 hr
17D-WN-RSS	2-R	0	0	0	0
	3-R	2	1	0	0
	4-R	3	0	0	0
	5-R	0	0	0	0
	6-R	0	0	0	0
	7-R	0	0	0	0
17D-WN	8-R	4	5	5	5
	9-R	3	2	1	0
	11-R	2	4	1	0
	12-R	2	5	1	0
	13-R	2	3	2	2
	14-R	2	3	2	0
RSS vaccine	16-R	3	0	0	0
	17-R	5	5	5	5
	18-R	4	5	4	4
	19-R	3	0	0	0
	20-R	2	5	2	2
	21-R	3	2	0	0
Controls	22-R	4	5	5	5
	23-R	2	5	5	5
	24-R	3	5	5	5
	25-R	5	5	5	5
	26-R	3	4	5	4
	27-R	3	5	5	5

experiments were carried out in which spider monkeys were given a series of injections consisting of 17D and West Nile virus plus one injection of killed Russian spring-summer virus. They were subsequently challenged subcutaneously with St. Louis encephalitis virus. These experiments were carried out in ways similar to those shown in Table 11. Table 14 shows that the vaccine gives some protection against St. Louis encephalitis virus under the experimental conditions, as indicated by a lowering of the amount of virus circulating in the blood and by the shorter duration of viremia.

Effect of the triple vaccination procedure on subsequent challenge with dengue virus, types 1 and 2, and Murray Valley virus. Some protection was given by the triple inoculation procedure against challenge with types 1 and 2 of dengue virus as well as Murray Valley encephalitis virus as shown in Tables 15 and 16. One thousand mouse i.c. LD_{50} were used in the dengue challenge tests and 1,000 mouse LD_{50} were also used in the Murray Valley experiments.

It should be pointed out that for protection against dengue types 1, 2, 3, and 4, St. Louis encephalitis virus, Murray Valley encephalitis, the two strains of Russian spring-summer virus complex, and Wesselsbron virus, it is not clear that the first inoculation with 17D must precede the West Nile-Russian spring-summer virus inoculations. However, 17D was used before

TABLE 12

Effect in chimpanzees of 17D-West Nile-Russian spring-summer triple vaccination on subsequent subcutaneous inoculation with either type 3 or type 4 dengue virus

Group	Challenge strain	Chimpanzee no.	Demonstration of viremia by intracerebral inoculation of suckling mice*		
			48 hr	72 hr	96 hr
17D-WN-RSS	Type 3 dengue	1-D	2	0	0
		2-D	3	1	0
		3-D	0	0	0
		4-D	0	0	0
17D-WN-RSS	Type 4 dengue	5-D	0	2	0
		6-D	0	1	0
		7-D	0	0	0
		8-D	0	2	0
Controls	Type 3 dengue	9-D	3	9	8
		10-D	10	10	8
		11-D	7	10	10
		12-D	4	9	10
Controls	Type 4 dengue	13-D	3	10	10
		14-D	7	10	10
		15-D	2	9	8
		16-D	3	10	10

* Whole blood diluted to 10%. For each interval 0.02-ml volumes were inoculated intracerebrally into 2 groups of suckling mice. A total of 10 suckling mice was used for each interval. Numbers represent specific deaths among the 10 mice.

giving the West Nile-Russian spring-summer virus inoculations because it does lower the titer of West Nile virus in the blood (Table 8), thus reducing the chances of causing clinical disease, and also increases the antibody titer of dengue type 1 virus as shown in Table 9.

Attenuation of the TL-443 West Nile isolate by passage in chick embryo tissue cultures. The results described above indicated that TL-443 strain was still too virulent to be considered for an experiment in man. Investigations were therefore undertaken to attenuate this TL-443 isolate. Specifically, the objective was to lessen the encephalitogenic potential and still retain the immunogenic potential. Twelve 7-day-old chick embryos were inoculated via the allantoic sac and incubated at 35°C. It was found that the virus reached its maximum concentration 4 to 5 days post inoculation. Titrations showed that the concentration of virus in the embryo was far higher than in the allantoic fluid or amniotic fluid. In making passages of virus incubated in embryos, the infected embryo was decapitated and its spinal cord removed by gross dissection. This was done in order to have less virus that may have multiplied in the brain and spinal cord. A 10% suspension was prepared by grinding the embryo in a Ten-Broeck tissue grinder in 10% inactivated normal rabbit serum; 0.1 ml of the 10% suspension was then inoculated into a new 7-day-old chick embryo.

After 10 such passages, chick embryo tissue cultures were used for further passages. Four tubes were inoculated with 0.1 ml of a 10% suspension made from each infected embryo. The cultures were 2 days old at the time they were seeded with virus. All virus dilutions were made in 10% inactivated, normal rabbit serum. The

TABLE 13

Viremia induced in chimpanzees by Wesselsbron virus following triple vaccination

Group	Chimpanzee no.*	Demonstration of viremia by intracerebral inoculation of suckling mice†		
		48 hr	72 hr	96 hr
17D-WN-RSS	42	2	1	0
	43	4	2	0
	44	2	2	0
	45	3	1	0
	46	0	0	0
	47	3	2	1
Controls	48	3	9	8
	49	10	10	4
	50	7	10	9
	51	3	4	0
	52	5	9	8
	53	4	10	9

* All chimpanzees were challenged subcutaneously with 8,000 suckling mice i.c. LD_{50}.

† Whole blood diluted to 10%. For each chimpanzee 0.02-ml volumes of diluted blood were inoculated intracerebrally into 2 groups of suckling mice. A total of 10 suckling mice was used for each interval for each animal. Numbers represent specific deaths among the 10 mice. The chimpanzees had no detectable neutralizing antibodies against Wesselsbron virus before receiving any virus inoculations.

TABLE 14

*Viremia induced in spider monkeys with St. Louis encephalitis virus following triple vaccination**

Group	Monkey no.	Demonstration of viremia by intracerebral inoculation of 8-10 gram mice			
		48 hr	72 hr	96 hr	120 hr
17D-WN-RSS	A	0	4	0	0
	B	0	0	0	0
	C	0	0	2	0
	D	6	0	1	0
	E	0	0	0	0
	F	0	0	0	0
Controls	G	4	10	10	2
	H	5	9	10	3
	I	4	10	9	2
	J	3	10	10	4
	K	2	10	9	2
	L	4	10	9	3

* Both groups of monkeys were challenged subcutaneously with 6,000 8- to 10-gram mouse i.c. LD_{50}. In determining viremia, whole blood was diluted to 10%. Ten mice were inoculated at each interval for each monkey. The sera of all monkeys used in the above experiments before inoculation with any virus specimens were negative for St. Louis encephalitis virus neutralization antibodies and other group B antibodies described in Methods.

cultures were allowed to incubate for 60 minutes at 34°C after which 0.9 ml of Hanks balanced salt solution was added to each tube and the cultures were incubated at 34°C on a roller drum. They were examined daily for cytopathogenic effect (CPE) and when approximately 75% of the cell layer in the tubes showed CPE, the fluids were harvested, quick-frozen, and stored at −70°C in sealed ampules. A 1 to 10 dilution of the fluids in 10% inactivated, normal rabbit serum was then used to inoculate new cultures. This procedure was continued for 18 passages.

At this time, plaque studies were undertaken using the method of Dulbecco and Vogt.[23] Minced chick embryo tissue was used in all these studies. Ten virus isolates prepared from various plaques were studied for their encephalitogenic capacity by inoculating various concentrations of the viruses intracerebrally into rhesus monkeys. The progeny of two plaques, numbers 2 and 4, were chosen for intensive study since graded doses of these viruses showed no ability to cause clinical signs of encephalitis when inoculated into rhesus monkeys. These isolates were purified further by three plaque passages. Table 17 shows a comparison of the various titers of the three reference strains, the TL-443 isolate and the two attenuated plaque purified isolates in various hosts.

A comparison of the encephalitogenic potential in rhesus monkeys of two reference strains of West Nile virus as compared to the original TL-443 isolate and the two plaque-purified TL-443 isolates is presented in Table 18. It is apparent that these latter two isolates have much less encephalitogenic potential in rhesus monkeys than does any West Nile virus yet isolated.

TABLE 15

Effect in chimpanzees of 17D-West Nile-Russian spring-summer triple vaccination on subsequent subcutaneous inoculation with either type 1 or type 2 dengue virus

Group	Challenge strain	Chimpanzee no.	Demonstration of viremia by intracerebral inoculation of suckling mice*		
			48 hr	72 hr	96 hr
17D-WN-RSS	Type 1 dengue	17-D	0	0	0
		18-D	0	1	0
		19-D	2	0	0
		20-D	0	1	0
		21-D	0	0	0
17D-WN-RSS	Type 2 dengue	22-D	0	0	0
		23-D	1	2	0
		24-D	0	0	0
		25-D	2	1	0
		26-D	0	0	0
Controls	Type 1 dengue	27-D	4	9	6
		28-D	6	10	7
		29-D	5	10	4
		30-D	3	10	8
Controls	Type 2 dengue	31-D	2	10	8
		32-D	7	10	9
		33-D	3	4	8
		34-D	8	4	9

* Whole blood diluted to 10%. For each interval 0.02-ml volumes were inoculated intracerebrally into 2 groups of suckling mice. A total of 10 suckling mice was used for each interval. Numbers represent specific deaths among the 10 mice.

TABLE 16

*Effect in chimpanzees of 17D West Nile-Russian spring-summer triple vaccination on subsequent subcutaneous inoculation with Murray Valley virus**

Group	Chimpanzee no.	No. of specific deaths in groups of 6 8–10 gram mice inoculated intracerebrally with 2% whole blood		
		48 hr	72 hr	96 hr
17D-WN-RSS	MV-1	2	0	0
	MV-2	1	0	0
	MV-3	0	1	0
	MV-4	2	0	0
	MV-5	0	0	0
	MV-6	0	0	0
Controls	MV-7	2	6	5
	MV-8	2	6	6
	MV-9	0	0	0
	MV-10	4	6	6
	MV-11	3	5	6
	MV-12	3	4	6
	MV-13	4	6	4

* The monkeys used in this test had no detectable neutralizing antibodies to Murray Valley encephalitis virus before being vaccinated. The control monkeys had no detectable neutralizing antibodies at time of challenge. None of the monkeys had hemagglutinating inhibiting antibodies to Murray Valley virus before being inoculated with any virus.

Histopathological studies of the brain and spinal cord of monkeys inoculated intracerebrally with these two isolates are in progress.* The titers in various systems of the original TL-443 isolate and the two plaque-purified isolates are shown in Table 19.

Table 20 shows the viremia produced by the Egypt 101 strain, the Lennette-Koprowski strain, the TL-443 West Nile isolate, and the plaque-purified attenuated TL-443 isolate number 4 in spider monkeys. It should be recalled that for man the Egypt 101 strain is the most

* These studies, carried out in collaboration with Dr. Oppenheimer of the Department of Pathology, School of Medicine, Johns Hopkins University, indicate that the attenuated strains of West Nile viruses cause very minimal lesions at the site of inoculation and no lesions in the spinal cord of the monkeys. This is a much different pathological finding from what was observed with the Egypt 101 strain and the Lennette-Koprowski strain.[15]

TABLE 17

*Comparison of the titers of the three reference strains, original TL-443 isolate and attenuated TL-443 plaque purified isolates in various hosts**

Virus strain	3-day-old mice		8–10 gram mice i.c.	7-day chick embryo yolk sac	6-week hamsters i.p.
	i.c.	i.p.			
Egypt 101	7.1	7.0	6.3	5.6	5.2
WN-1	8.2	8.1	7.4	6.9	7.9
Len-75	7.9	7.7	7.6	7.2	3.2
TL-443	7.9	7.3	7.4	6.1	3.1
No. 2 plaque of TL-443	7.8	7.5	7.2	6.6	3.2
No. 4 plaque of TL-443	7.8	7.4	7.3	6.7	3.0

* All titrations carried out as described in Table 1.

virulent and the Lennette-Koprowski is avirulent.[2] However, not only does the Lennette-Koprowski isolate produce no clinical disease in man but it also has little immunogenic capacity.[2] This is not surprising in view of its low peripheral infectivity for primates. In spider monkeys, the attenuated plaque 4 isolate showed a viremia similar to the parent isolate TL-443.

Table 21 shows that a previous inoculation with 17D yellow fever vaccine reduces the viremia produced on subsequent challenge with the attenuated plaque 4 isolate, although only 2 of 7 spider monkeys were completely protected. However, even in those yellow fever vaccinated monkeys in which viremia occurred after inoculation of West Nile virus, it was of shorter duration and the virus titer was lower. This would seem to support the use of 17D vaccine before giving the attenuated West Nile virus as discussed earlier in this paper.

Since the evidence of the reduction in encephalitogenic potential of the attenuated plaque-purified West Nile isolates was clear, the next question was whether such attenuated strains still possessed suitable immunizing capacity. Experiments were therefore set up comparable to the experiment shown in Table 9. From the results shown in Table 22, it is apparent that the attenuated plaque 4 isolate of West Nile virus still possessed immunizing ability similar to the original parent type. This experiment was repeated once more with results similar to those shown in Table 22.

TABLE 18
Intracerebral pathogenicity of various West Nile isolates in rhesus monkeys

Virus strain	Virus dose*	Deaths due to encephalitis	No. showing signs of encephalitis†/no. inoculated
Egypt 101	1,000	1/5	4/5
	100,000	5/5	5/5
Lennette-75	1,000	0/5	0/5
	100,000	0/10	7/10
TL-443	5,000	0/5	3/5
	100,000	1/5	5/5
No. 2 plaque purified TL-443	5,000	0/5	0/5
	100,000	0/10	0/10
No. 4 plaque purified TL-443	5,000	0/5	0/5
	100,000	0/10	0/10
	1,000,000	0/25	0/25

* Dose given in terms of 8- to 10-gram mouse i.c. LD_{50} inoculated intracerebrally into each group.

† Signs observed were the following: trembling, incoordination, clumsiness, irritability, weakness, ataxia, roughing of hair, loss of appetite, spasticity, paralysis of limbs, coma, convulsions, and unusual eye movements. In all groups recorded as negative, none of the above signs of encephalitis were noted, nor were any other signs of illness noted. The animals receiving the attenuated isolates did not show temperatures of over 40°C on any day during the 30-day observation period. Temperatures of over 40°C were observed in animals receiving the other isolates.

Table 23 shows a comparison of the encephalitogenic potential, the immunogenic capacity, and the peripheral infectivity of various West Nile isolates in various hosts. It is not possible at this time to compare the properties of all isolates in relation to their virulence for man, since the two attenuated TL-443 isolates have not been studied in human beings at this time. However, it is possible that the encephalitogenic capacity of an isolate in rhesus monkeys may be the best laboratory criterion available at the present time of the ability of an isolate to produce encephalitis in man. It should be remembered that the three reference strains were studied only in cancer patients.

Effect of passing attenuated plaque number 4 TL-443 isolate intracerebrally in rhesus monkeys and suckling mice on the encephalitogenic ability of the virus. Table 24 shows that after attenuated plaque number 4 had been passed 11 times intracerebrally in rhesus monkeys, alternating every monkey passage with an intracerebral passage in suckling mice, it still retained its low encephalitogenic capacity for monkeys. When the 11th monkey passage virus was purified by the plaque technique and seven clones tested intracerebrally in rhesus monkeys, all seven clones showed the same low encephalitogenic ability as the original attenuated plaque number 4 clone. These experiments were carried out by making a 20% suspension of the whole monkey brain 5 days after inoculation. The suspension was clarified by centrifugation at 2000 X G for 15 minutes. The supernatant fluid was harvested and 3 8- to 10-gram mice inoculated with 0.03 ml volumes. When the mice were moribund, their brains were harvested and pooled, a 1 to 10 dilution made of a 10% suspension, and 0.3 ml of this material was inoculated intracerebrally into a rhesus monkey. This whole procedure was repeated 10 more times. The mice had to be used for alternate passages, since the attenuated strain was lost on continual passage by intracerebral inoculation in rhesus monkeys.

Six subcutaneous passages in spider monkeys were also made in the above manner except that instead of using the brain suspension, blood from the spider monkey was taken 72 hours after inoculation, diluted to 20%, and inoculated into mice. Similar passages were also carried out in 4-day-old White Rock chickens. With both spider monkeys and chickens, mice had to be used for alternate passages for the same reasons as in the rhesus monkey experiment described above. Passing the virus in these latter two systems did not change the encephalitogenic ability of the virus as Table 24 shows. The number of clones tested in the spider monkey and chicken experiments was the same as that tested in the rhesus monkey experiments. All these data indicate that this isolate is stable and will not easily revert to a more virulent form.

The passage of isolate TP-21 of the Russian spring-summer virus complex in chick embryo tissue cultures. It has been mentioned earlier in this paper that the data indicate that the best vaccine against group B viruses would consist of

attenuated strains of Japanese B virus and a virus from the Russian spring-summer complex to be given along with the 17D virus. Studies on the attenuation of Japanese B virus carried out in this laboratory have as yet not been successful. However, the TP-21 strain isolated by Smith[24] in Malaya, which belongs to the Russian spring-summer group of viruses, appears to possess

TABLE 19

*Various properties of TL-443 West Nile isolate and the two attenuated plaque purified isolates of TL-443**

Virus	TCD_{50}†	3-day-old mouse i.c. LD_{50}	8–10 gram mouse i.c. LD_{50}	7-day-old mouse i.p. LD_{50}	8–10 gram mouse i.p. LD_{50}	PFU‡	HA§
Original TL-443	7.5	9.0	9.0	8.5	6.5	0‖	2048
Plaque 2	7.5	9.0	8.0	7.0	6.25	4×10^7	1024
Plaque 4	7.5	9.0	8.5	8.5	6.5	8×10^8	1024

* Titers are all reported for 1-ml volume.
† TCD_{50}—tissue culture infective doses.
‡ PFU—plaque forming units.
§ HA—hemagglutination titer.
‖ 0—indicates this virus could not form plaques under the experimental conditions.

TABLE 20

Viremia induced in 2- to 4-pound spider monkeys (Ateles geoffroyi) *following subcutaneous inoculation with various West Nile isolates*

Virus strain and dose	Monkey no.	Demonstration of viremia by intracerebral inoculation of 8–10 gram mice*																	
		24 hr			48 hr			72 hr			96 hr			120 hr			144 hr		
		25%	10%	1%	25%	10%	1%	25%	10%	1%	25%	10%	1%	25%	10%	1%	25%	10%	1%
Egypt 101, 3,000 8–10 gram mouse i.c. LD_{50}	1	0	0	0	0	0	0	1	0	0	3	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	4	4	2	4	2	0	4	2	0	0	0	0
	4	0	0	0	4	1	0	4	2	0	3	0	0	1	0	0	0	0	0
	5	0	0	0	3	0	0	3	1	0	4	1	0	4	0	0	1	0	0
Lennette-75, 5,000 8–10 gram mouse i.c. LD_{50}	1	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	2	0	0	2	1	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TL-443, 7,000 8–10 gram mouse i.c. LD_{50}	1	1	0	0	0	0	0	1	0	0	4	3	1	2	0	0	0	0	0
	2	2	0	0	2	0	0	4	3	1	4	3	1	4	2	0	0	0	0
	3	0	0	0	4	2	0	4	2	0	4	2	1	4	0	0	2	0	0
	4	0	0	0	1	0	0	4	4	2	2	0	0	2	0	0	2	0	0
	5	0	0	0	1	0	0	4	3	1	4	3	1	4	2	0	1	0	0
No. 4 plaque purified TL-443, 6,600 8–10 gram mouse LD_{50}	1	4	1	1	2	2	1	4	2	0	2	0	0	0	0	0	0	0	0
	2	4	4	0	2	0	0	1	1	0	2	2	1	0	0	0	0	0	0
	3	3	4	4	4	1	0	2	0	0	3	0	0	0	0	0	0	0	0
	4	2	4	0	2	2	1	1	0	0	4	2	1	0	0	0	0	0	0
	5	0	4	1	3	1	0	0	0	0	3	2	1	0	0	0	0	0	0

* Whole blood diluted to 25%, 10%, and 1% concentrations; 0.03-ml volumes inoculated intracerebrally into groups of 4 8- to 10-gram mice. Numbers represent specific deaths among each group of 4 mice inoculated.

TABLE 21

Effect of 17D vaccine on viremia produced in spider monkeys by subsequent subcutaneous inoculation with attenuated plaque 4 isolate

Virus strains inoculated	Monkey no.	Demonstration of viremia by intracerebral inoculation of 8–10 gram mice*						
		48 hr		72 hr		96 hr		120 hr
		20%	2%	20%	2%	20%	2%	20%
$10^{5.1}$ mouse i.c. LD_{50} of 17D	51	2	0	2	0	0	0	0
followed 2 months later by	52	1	0	0	0	0	0	0
1,000 mouse i.c. LD_{50} of	53	1	0	2	0	0	0	0
plaque 4 isolate	54	2	0	4	1	1	0	0
	55	0	0	2	0	1	0	0
	56	0	0	0	0	0	0	0
	57	0	0	0	0	0	0	0
1,000 mouse i.c. LD_{50} of plaque	58	2	0	4	2	2	0	2
4 isolate	59	1	0	4	1	2	1	2
	60	2	0	3	2	2	0	0
	61	2	0	4	3	2	0	0
	62	2	0	4	2	2	0	2

* Whole blood diluted to 20% and 2%; 0.03-ml volumes inoculated intracerebrally into groups of 4 8- to 10-gram mice. Numbers represent specific deaths among each group of 4 mice inoculated.

TABLE 22

*Neutralization indices of pre- and post-experimental sera of spider monkeys used in 17D-attenuated plaque 4 West Nile virus experiment**

Experimental group	No.	Pre-experimental neutralizing index of sera				Neutralizing index of sera								
						Post 17D-pre-West Nile virus				Post-attenuated West Nile virus				
		WN†	JB	YF	D1	WN	JB	YF	D1	WN	JB	SL	YF	D1
A. 17D plus attenuated plaque 4 (monkeys	1	0	0	0	0	0	0	3.9	2.1	3.8	2.1	1.3	4.0	2.9
given 17D; titer $10^{4.1}$ i.c. LD_{50} in 8–10	2	0	0	0	0	0	0	2.7	0	4.1	2.1	1.6	4.5	2.4
gram mice. Two months later 10,000 i.c.	3	0	0	0	0	0	0	3.5	1.3	3.6	1.8	0	4.1	1.9
mouse LD_{50} given subcutaneously of	4	0	0	0	0	0	0	3.2	0	3.7	1.9	1.4	4.5	2.9
plaque 4	5	0	0	0	0	0	0	3.7	1.8	3.9	2.6	1.6	4.2	2.5
B. Monkeys given 10,000 8–10 gram mouse	6	0	0	0	0					3.1	1.9	0	0	1.0
i.c. LD_{50} of plaque 4 West Nile virus	7	0	0	0	0					3.2	1.7	1.5	0	1.4
	8	0	0	0	0					3.7	2.1	1.6	0	1.2
	9	0	0	0	0					3.2	1.9	1.3	0	0
	10	0	0	0	0					3.0	1.7	1.0	0	1.1
	11	0	0	0	0					3.4	1.9	0	0	1.0

* Detectable neutralizing antibodies for St. Louis encephalitis virus were lacking in all sera of monkeys taken before inoculation.

† WN represents West Nile virus; JB—Japanese B; SL—St. Louis; YF—yellow fever (17D); D1—dengue virus strain 1 (Hawaiian).

TABLE 23

*Comparison of encephalitogenic capacity, peripheral infectivity, and immunogenic ability of various West Nile isolates**

Strain	Virulence for man	Encephalitogenic capacity in rhesus monkeys	Immunogenic capacity in spider monkeys	Peripheral infecting capacity in		
				Spider monkeys	Chicks	Hamsters
Egypt 101	++	++++	++++	++++	++++	++
WN-1	+	++	++	++	++	++++
Lennette	—	++	—	+	+	+
TL-443	Unknown	++	++++			+
Attenuated plaque 4 purified TL-443	Unknown	—	++++	++++	+++	+

* The various properties of the different West Nile isolates were graded on their ability to produce the various studied responses with graded doses of virus. ++++ means that the ability of the isolate to produce the effect is obtained with relatively small amounts of virus. Peripheral infectivity was measured by the intraperitoneal route or subcutaneously. In hamsters it was determined by the death of the hamsters, and in chicks it was determined by the number of 1-week-old chicks infected when inoculated with graded doses of virus and the maximum viremia produced.

TABLE 24

Effect of passage in various hosts on the encephalitogenic ability of plaque 4

Virus	Virus dose inoculated intracerebrally into rhesus monkeys*	No. of monkeys showing any clinical signs of encephalitis/no. inoculated
Original plaque 4	200,000	0/12
Plaque 4 after 11 alternate intracerebral passages in monkeys and suckling mice	300,000	0/12
Plaque 4 after six alternate subcutaneous passages in spider monkeys and intracerebral passages in suckling mice	600,000	0/12
Plaque 4 after six alternate subcutaneous passages in 4-day-old chicks and intracerebral passages in suckling mice	800,000	0/12

* Dose given in terms of 8- to 10-gram mouse i.c. LD_{50}.

characteristics which might make it suitable for further study.

This virus was passed 9 times in chick embryos and then passed 38 times in chick embryo tissue cultures by methods exactly like those described for the attenuation of West Nile virus. The only difference was that the virus was harvested at the end of 72 hours in chick embryo tissue cultures rather than at the end of 48 hours as in the West Nile experiments. Ten plaques were selected for study in rhesus monkeys. One of the plaques yielded a virus isolate which produced no signs of encephalitis, according to the standards discussed in Table 18, when 300,000 8- to 10-gram mouse i.c. LD_{50} were inoculated intracerebrally into rhesus monkeys. Histopathological studies of 10 monkeys showed no signs of pathology in the central nervous system and very minimal signs of pathology at the site of inoculation. Table 25 shows a comparison of the encephalitogenic properties of the parent strain of TP-21

TABLE 25

Encephalitogenic effect of TP-21 before and after passage in chick embryo tissue cultures as determined by intracerebral inoculation into rhesus monkeys

Strain inoculated	No. of monkeys inoculated	Dose of virus inoculated*	No. showing encephalitis†
Parent TP-21	6	20,000	2/6
Chick embryo passage TP-21-9	18	300,000	0/18

* Virus dose given in terms of 8- to 10-gram mouse i.c. LD_{50}.

† Same criteria of encephalitis as described in Table 18.

TABLE 26

Effect of the TP-21-9 isolate in spider monkeys on subsequent challenge with various viruses of the Russian spring-summer virus complex

Experiment group	Challenge virus*	Monkey no.	Viremia as determined by intracerebral inoculations into 8–10 gram mice†			
			48 hr	72 hr	96 hr	120 hr
17D-WN-TP-21-9	Russian spring-summer encephalitis	41	3	1	0	0
		42	0	0	0	0
		43	0	0	0	0
		44	4	1	0	0
		45	2	1	0	0
17D-WN-TP-21-9	Omsk	46	2	0	0	0
		47	0	0	0	0
		48	4	1	0	0
		49	0	0	0	0
		50	0	0	0	0
17D-WN-TP-21-9	Central European encephalitis virus	51	3	2	0	0
		52	0	0	0	0
		53	0	0	0	0
		54	3	0	0	0
		55	0	0	0	0
17D-WN-TP-21-9	Kyasanur Forest virus	56	0	0	0	0
		57	0	0	0	0
		58	3	0	0	0
		59	0	0	0	0
		60	1	0	0	0
Control	Russian spring-summer encephalitis	61	8	9	9	8
		62	6	18	10	10
		63	5	9	10	9
		64	5	10	10	7
		65	4	10	9	10
Control	Omsk	67	4	10	10	9
		68	5	10	10	10
		69	0	10	10	10
		70	2	10	10	10
		71	3	8	5	9
Control	Central European encephalitis virus	72	2	9	4	10
		73	3	10	7	10
		74	4	10	10	8
		75	3	10	10	9
		76	2	8	10	9
Control	Kyasanur Forest virus	77	4	7	6	3
		78	2	4	10	5
		79	5	10	7	8
		80	6	10	8	10
		81	4	10	9	6

* Approximately 300 8- to 10-gram mouse i.c. LD_{50} were used for all challenges. The monkeys given TP-21-9 were inoculated subcutaneously with approximately 50,000 mouse i.c. LD_{50}.

† Blood was diluted to 1% and inoculated intracerebrally into groups of 10 8- to 10-gram mice for each time interval.

as compared to the isolate after passage in chick embryos and chick embryo tissue cultures when both isolates were inoculated intracerebrally into rhesus monkeys. The isolate TP-21-9 was purified by three further plaque isolations. The viremia induced in spider monkeys by subcutaneous inoculation of TP-21-9 is of low titer (not over 1.5 logs by intracerebral tests in 8- to 10-gram mice) and lasts about 4 days. This statement is based on the study of six spider monkeys inoculated subcutaneously with approximately 7,000 mouse i.c. LD_{50}.

Table 26 shows that if TP-21-9 is substituted for the one inoculation of Russian spring-summer encephalitis vaccine in the triple vaccination procedure, it will protect some spider monkeys to a considerable extent against challenge with virulent Russian spring-summer encephalitis virus, Moscow strain, an Omsk strain of the Russian spring-summer complex, a Central European strain of the Russian spring-summer complex, as well as the etiological agent of Kyasanur Forest disease, which is also a member of the Russian spring-summer complex. The monkeys were challenged 2 months after receiving the TP-21-9 isolate. It would appear that the best experimental vaccine against group B viruses at the present time found in this laboratory would consist of 17D virus, plus the attenuated plaque 4 isolate of West Nile virus discussed earlier in the paper, plus the TP-21-9 isolate of the Russian spring-summer complex. Preliminary data indicate that TP-21-9 isolate alone will give some protection against members of the Russian spring-summer complex.

Studies of the growth of the attenuated West Nile virus in mosquito vectors are in progress with Dr. L. Rozeboom and will be described in a separate publication. These experiments have shown that the attenuated strain of West Nile virus multiplies very poorly in the three mosquito vectors studied, giving titers approximately 10,000-fold less than those of the parent strain.

DISCUSSION

In order to study the virulence of the West Nile virus, it was necessary to define clearly the various characters shown by the different West Nile isolates and to study each in a quantitative manner. The important characters that were studied in various laboratory animals were encephalitogenic ability (the ability to cause encephalitis when the viruses were inoculated intracerebrally into rhesus monkeys), peripheral infectivity (the capacity to produce viremia when the viruses were inoculated intraperitoneally or subcutaneously), and the immunogenic capacity (the ability to produce heterologous group B neutralizing antibodies when inoculated by peripheral routes).

The data presented in this paper clearly show that the different West Nile isolates may vary in their pathogenicity for various laboratory animals, thus confirming and extending previous reports from this laboratory.[15]

By passage in chick embryo tissue culture, it has been possible markedly to reduce the encephalitogenic potential of a strain of West Nile virus obtained from Israel. Indeed, no signs of encephalitis were noted when high concentrations of the attenuated viruses were inoculated intracerebrally into rhesus monkeys. This concentration was at least 1,000 times that necessary to produce encephalitis with the parent type, since the parent isolate gave signs of encephalitis with 1,000 mouse i.c. LD_{50} and no signs of encephalitis were noted when rhesus monkeys were inoculated intracerebrally with 1,000,000 mouse i.c. LD_{50} of the attenuated isolate. This attenuated strain, which has been purified by the plaque technique, would seem to warrant further intensive studies for possible use in man, since in comparison with the three reference strains it would appear to have some desirable biological properties for a vaccine, *i.e.*, extremely low encephalitogenic activity in rhesus monkeys but enough immunogenic capacity to produce neutralizing antibody against itself and low levels against Japanese B virus, as well as sensitization of the immune mechanisms of the host to the other viruses used in the triple vaccination procedure. It has been found that spider monkeys inoculated with 17D vaccine show a lower viremia of shorter duration when challenged with the attenuated West Nile virus than do control spider monkeys. This may decrease the possibility of this isolate causing clinical disease in man, since in the triple vaccination procedure 17D yellow fever vaccine would be given before the attenuated West Nile virus.

Study of virulence characters of West Nile viruses has shown that the encephalitogenic potential for rhesus monkeys appears to be distinct from the property of the virus which deter-

mines peripheral infectivity in spider monkeys.

Other data presented in the paper show, on the basis of either the lowering of viremia or the development of neutralizing antibodies, that primates given a combination of 17D-yellow fever vaccine, followed by an injection of living West Nile virus, followed by one injection of formalin-inactivated Russian spring-summer virus vaccine, will show some resistance against the West Nile-Murray Valley-St. Louis encephalitis-Japanese B encephalitis group of viruses as well as against dengue viruses types 1, 2, 3, and 4, the two strains of the Russian spring-summer virus complex that were tested, and the Wesselsbron virus. The extent and duration of this immunity and related problems will be discussed in a later paper.

A strain of the TP-21 virus of the Russian spring-summer virus complex was passed a number of times in chick embryo tissue cultures. The progeny from one virus plaque was further purified by the plaque technique. This isolate caused no encephalitis when inoculated intracerebrally into rhesus monkeys in a concentration of approximately 300,000 mouse i.c. LD_{50} and gave a low viremia when inoculated subcutaneously into spider monkeys.

In the triple vaccination procedure, if this isolate of TP-21 was substituted for the formalin-inactivated Russian spring-summer encephalitis vaccine, considerable protection was observed when spider monkeys were challenged subcutaneously with the Moscow strain of Russian spring-summer encephalitis, an Omsk strain of the Russian spring-summer virus complex, a Central European encephalitis virus isolate, or an isolate of Kyasanur Forest virus.

SUMMARY

Spider monkeys and chimpanzees were given a series of three injections consisting of 17D yellow fever virus, followed by living West Nile virus, followed by a third injection which consisted of formalin-inactivated Russian spring-summer virus vaccine. On the basis of neutralizing antibody responses, the limitation of viremia, or both, developing when the animals were challenged with virulent viruses, these primates were judged to be protected to a considerable extent against Japanese B encephalitis, West Nile virus, St. Louis encephalitis, Murray Valley encephalitis virus, dengue types 1, 2, 3, and 4, two antigenic types of the Russian spring-summer virus complex, and Wesselsbron virus.

An isolate of West Nile virus was passed a number of times in chick embryo tissue cultures and purified by the plaque technique. The progeny of two virus plaques, in a concentration of 10^6 mouse intracerebral lethal doses, did not produce encephalitis in intracerebrally inoculated rhesus monkeys. These attenuated viral preparations, on the basis of intracerebral titrations in mice, had at least 1,000 times the virus concentration that was necessary to produce encephalitis with the parent type. One of these attenuated isolates still produced homologous and heterologous neutralizing antibodies comparable to those of the parent strain. The data indicate that this attenuated West Nile virus did not revert to a more virulent form after alternate intracerebral passages in rhesus monkeys and suckling mice.

The TP-21 strain of the Russian spring-summer virus complex was passed a number of times in chick embryo tissue cultures and purified by the plaque technique. The progeny from one of the virus plaques, in a concentration of approximately 300,000 mouse i.c. LD_{50}, did not produce encephalitis when inoculated intracerebrally into rhesus monkeys. When this purified virus isolate of TP-21 was substituted for the formalin-inactivated Russian spring-summer vaccine in the triple vaccination procedure, considerable protection was noted in spider monkeys challenged with four members of the Russian spring-summer group of viruses.

ACKNOWLEDGMENTS

Some of the experiments in this paper were carried out by Drs. James Ganaway and James Parks. The senior author wishes to thank Drs. Max Theiler and Joseph Smadel for their encouragement during the studies of arthropod-borne viruses carried out in this laboratory during the last 7 years. The very early studies in this laboratory were started after Dr. Theiler pointed out to the senior author the possible importance of immunological overlapping in the natural history of arthropod-borne viruses. We wish to thank Drs. Theiler, Smadel, Albert Sabin, Jordi Casals, Loring Whitman, Delphine Clark, and Philip E. Sartwell for reading this manuscript and giving us their comments.

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AN ACID-FAST PATHOGEN OF FRESH-WATER SNAILS*

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Acid-fast bacilli, sometimes pathogenic, have frequently been reported from cold-blooded vertebrates.[1] Except for a brief note[2] describing the presence of acid-fast organisms in sections of coleopterous insects, little is known of the association of such bacteria with invertebrate hosts. The presence of an acid-fast bacillus in tissue sections of the aquatic pulmonate snail, *Australorbis glabratus*, was first reported by Pan.[3] Subsequently, we recognized a similar, if not identical, organism in laboratory-reared *Helisoma anceps*, from which pure cultures were successfully established. The organism is a species of *Mycobacterium*; and, so far as we are aware, this represents the first such isolation from an invertebrate. The present study was initiated as one phase of a program directed towards the biological control of the snail vectors of schistosomiasis.

MATERIALS AND METHODS

Snail colonies. The colony of *H. anceps*, which has served as a source of infected snails and from which the bacillus was originally isolated, has been maintained in our laboratory since 1954. The history of this colony is not known except that the snails were maintained in a tropical fish aquarium for some years prior to 1954.

Thirteen strains of snails, representing 10 species, were used in transmission experiments. Table 1 lists the specific names, origin, and period of maintenance of the various snail strains. Details of maintenance of the various aquatic snails are essentially as have been described for the PR-1 strain of *A. glabratus*.[4] The two species of terrestrial snails, *Otala lactea* and *Helix aspersa*, were maintained in a terrarium made from a glass-sided aquarium similar to that used for the aquatic snails. A 3-inch substrate of autoclaved potting soil was provided and the snails were fed a diet of boiled lettuce.

Since spontaneous infections occurred in our stock colonies of *A. glabratus* and *Biomphalaria pfeifferi*, it was necessary to develop special colonies which could be used in transmission experiments. Egg masses were collected from stock colonies of *A. glabratus* and *B. pfeifferi* and surface treated with Clorox in accordance with the technique developed by Chernin.[5] The egg masses were than placed in 8-liter battery jars containing autoclaved aquarium water and allowed to hatch. These colonies were maintained in a room isolated from the regular stock colonies and snails selected at random were examined histologically and bacteriologically for the presence of the acid-fast organism. Repeated examinations failed to disclose the presence of acid-fast bacilli in snail tissues or in cultures thereof.

Isolation and cultivation of the bacillus. All isolations of the bacillus from snail tissues were made by a slight modification of the MacNabb technique.[6] The entire snail (minus the alimentary canal), or selected tissues, was homogenized in a sterile mortar containing 2 ml of sterile distilled water. The homogenate was transferred to a sterile test tube to which was added 2 ml of 3% HCl, plus a drop of bromocresol purple indicator. The contents of the tube were mixed by shaking and allowed to stand at room temperature (25°C) for 2 hours. Three per cent NaOH was added until the mixture became slightly alkaline. It was then centrifuged at 1,500 rpm for 15 minutes and the sediment used to inoculate tubes of Petragnani and Dorset egg media. Culture tubes were maintained at 25° and 37°C for 30 days. Positive cultures were stored at 5°C for 30 days and then routinely transferred.

Transmission studies. Four methods were used in attempts to pass the organism experimentally into different snail species. These included (1) dispersal of the organism into the aquatic medium (environmental contamination); (2) introduction of infected snails into an uninfected colony; (3) inoculation of bacilli into blood sinuses and tissues of normal snails; and (4) introduction of bacteria into the foot of normal snails by stab wounds. Inocula for these studies

* This investigation was supported (in part) by a research grant (E-513) and a training grant (2E-46) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service.

were prepared either by scraping 14-day-old Petragnani cultures and suspending the organisms in 2 to 3 ml of sterile distilled water, or by harvesting the organisms from 7-day-old Dubos' albumin broth (Difco) cultures which had been inoculated from 14-day-old Petragnani cultures. In the latter case, the cultures were centrifuged (1,500 rpm/15 min), washed 3 times with sterile distilled water, and then resuspended in 2 to 3 ml of sterile distilled water.

In experiments in which the bacilli were dispersed into the aquatic environment, 2 to 3 ml of inocula was placed in a finger bowl containing 100 ml of sterile distilled water and 5 to 10 snails were added. After 24 hours the snails and the contents of the finger bowl were added to a 3-liter battery jar containing 2.5 liters of autoclaved aquarium water. The battery jars were covered with Saran Wrap, continually aerated; and boiled lettuce was added as food at weekly intervals. Control snails were treated in a similar manner, but were not exposed to the organisms. Transmission of the bacilli was also attempted by the introduction of known infected snails into normal colonies in 3-liter battery jars maintained as described above.

In order to inject bacilli into them, the aquatic snails were first relaxed with urethane and then extended in accordance with a technique previously described.[7] Approximately 0.05 ml of inocula was introduced into each snail by means of a 0.25-cc tuberculin syringe fitted with a 30-gauge needle. Controls were injected with a similar volume of distilled water.

Stab wounds were made with a 25-gauge needle which had first been immersed in a 2-ml suspension of the bacilli. Controls were wounded with a sterile needle. After inoculation or wounding, snails were maintained in 3-liter battery jars as in other experiments.

Histopathologic studies. Tissues were routinely fixed in Newcomer's solution, although Zenker's, 5% formic acid, 10% neutral formalin, and Maximow's fixative were used on occasion. Embedding was done in paraffin and sections cut at 7 to 12 μ. Sections were stained with Lillie-Mayer's hemalum and eosin, hemalum and azure II-eosin, or Fite's carbol fuchsin with Janus green, acid hemalum, or methylene blue counterstains.[8] The histopathologic observations were based on examination of serial sections from 30 snails and on sections of selected tissues from an additional 25 snails.

BACTERIOLOGIC AND HISTOPATHOLOGIC OBSERVATIONS

Characteristics of the Mycobacterium. The organism is an acid-fast, alcohol-fast, gram variable bacillus, measuring 2.5 to 5.5 μ in length and 0.3 to 0.4 μ in width. Preparations made from organisms grown in Dubos' albumin broth, wet-fixed with osmium tetroxide fumes and stained with carbol fuchsin cold stain,[9] disclose distinct crossbandings along the entire length of the bacillus. Rarely, similar bandings may be observed in specimens stained by the Ziehl-Neelson method. The bacillus will grow on a variety of media such as Petragnani's, Dorset egg medium, Dubos' albumin broth, nutrient agar, blood agar, and brain-heart infusion agar. Strains were routinely maintained on Petragnani's medium, on which the organisms predominantly form smooth, discrete, droplet-like colonies, though occasionally rough, spreading forms have been noted. The organisms are highly chromogenic and colonies vary from light to golden yellow with the age of the culture. A brick-red mutant was noted which proved to be unstable since it often reverted to the original type on subculture. Growth occurred at temperatures ranging from 18° to 40°C with the optimum at 25° to 28°C. Original isolations on Petragnani's medium (25°C) yielded visible growth of minute droplet colonies in 9 to 12 days, and on nutrient agar (25°C), in 13 to 15 days. The organism produced acid in litmus milk, reduced nitrates to nitrites after 24 hours in Difco nitrate broth, and produced indol (Kovac's reagent) after 24 hours in 1% Difco tryptone solution.

Cultures of the bacillus have been sent to several specialists in an attempt to determine its specific identity, but at present its relationship to known species of *Mycobacterium* remains uncertain.

Gross and histopathologic changes in the snail host, H. anceps. Several manifestations of disease caused by the acid-fast organism were grossly observable in living snails, particularly in albino strains. The earliest indication of infection was a prominent clubbing of the tips of the tentacles, generally accompanied by a localized increase in pigmentation (Figure 1). Microscopically, the tips of the tentacles showed marked aplasia with large vacuolated areas (Figure 2). The increase in pigmentation was characterized by aggregates of pigment particles in the swollen tip region or

directly adjacent to it. The outer epithelium, which is normally columnar, appeared compressed and squamose. Loss of cilia frequently occurred. Small, spherical, refractile, yellow bodies were observable in the tissues of infected snails (Figures 1A and 1D) and could even be detected through the shell of the snail. Histological study of sections revealed that these yellow bodies were discrete lesions containing numerous acid-fast bacilli (Figures 2 and 3). The yellow bodies in snail tissues were always associated with tubercle-like lesions and their presence appeared to be pathognomonic. In long-standing infections tubercles developed on the surface epithelium with concurrent atrophy of the tentacles.

The organisms occur as intracellular parasites, particularly in the amebocytes (Figure 4). Parasitized amebocytes accumulate in small aggregates with other types of infected cells and are subsequently walled off by fibroblasts to form tubercles (Figure 5). Immature tubercles are composed primarily of masses of infected cells. As the tubercles mature, masses of necrotic cells become evident; however, these are absorbed and the mature tubercle becomes vacuolated and contains a massive conglomerate of bacilli (Figure 6). Bacilli appear to invade the fibroblasts forming the wall of the tubercle and thus spread the infection to surrounding tissue. As many as 200 lesions have been noted in serial sections of a single snail. Lesions were most frequently observed in the kidney, tentacles, rectal ridge, and foot, but were also seen in tissues of all the major organs (Figure 7). Although the preceding description is based primarily upon specimens of *H. anceps*, it is generally applicable to infections in the other species of Planorbidae used in this study.

SPONTANEOUS AND EXPERIMENTAL TRANSMISSION

Observations on spontaneous transmission of the bacillus were of necessity limited to periodic checks of the infected colony of *H. anceps*. At 6-month intervals, all snails in the colony were examined under a stereoscopic microscope (×13) for the presence of yellow bodies. Prevalence rates, based on gross detection of yellow bodies, reflect lower rates of infection than actually occur

TABLE 1

Origin and period of maintenance prior to exposure of snail strains used in transmission experiments

Species and strains	Origin	Time maintained prior to exposure*
Helisoma anceps	Brookline, Mass.	0–15 days
Helisoma trivolvis	?†	5 days
Helisoma trivolvis fallax (1)	Waltham, Mass.	2 days
Helisoma trivolvis fallax (2)	Brookline, Mass.	0 days
Australorbis glabratus (PR-1)	Puerto Rico‡	1–3 months
Australorbis glabratus (M)	Brazil§	14–60 days
Biomphalaria pfeifferi	Egypt‖	1–3 months
Bulinus truncatus	Egypt	4 months
Lymnaea palustris	?¶	2 years
Physa heterostropha	Brookline, Mass.	2 years
Marisa cornuarietis	Puerto Rico**	3.5 years
Helix aspersa	North Africa†	3–4 months
Otala lactea	North Africa†	3–4 months

* Refers only to maintenance in our laboratory.

† Obtained commercially.

‡ The strain was originally received from Dr. Redginal Hewitt, The Lederle Laboratories. A special colony derived from PR-1 strain was used in the experiments.

§ Received originally from Drs. Elmer Berry and Louis Olivier, Laboratory of Tropical Diseases, National Institutes of Health.

‖ The strain was originally received from Dr. Elmer Berry. A special colony derived from this strain was used in the experiments.

¶ Received originally from Sister Mary St. John Keefe, Emmanuel College.

** Received originally from Dr. Harold Harry and other members of the Army Tropical Research Laboratory, San Juan, Puerto Rico.

since early infections and deep tissue lesions could not be observed. The size of the population remained at a relatively constant level during the 3-year period of observation (Table 2). Spontaneous infection also occurred in stock colonies of *A. glabratus* and of *B. pfeifferi*, among which it was estimated that the infection rate ranged from 5 to 15%. It is not known what mechanisms are involved in the spontaneous transmission of this infection. It is of interest that acid-fast bacilli cultured from feces obtained from the rectum and hindgut of *H. anceps* produced characteristic lesions when inoculated into normal snails. Although fecal pollution may aid in maintaining the organism in the aquatic environment, it is suggested that the major source of environmental contamination may occur as the result of the decomposition of dead snails. Most aquatic snails will act as scavengers, and infection resulting from the ingestion of infective tissue seems possible.

TABLE 2

Prevalence of mycobacterial infection in a colony of Helisoma anceps, *1957–1960*

Date	Total no. snails	No. snails infected*	% infected
Feb. 1957	75	35	46.7
Aug. 1957	55	22	40.0
Feb. 1958	65	20	30.8
Aug. 1958	83	24	27.7
Feb. 1959	75	38	50.7
Aug. 1959	65	11	15.4
Feb. 1960	58	5	8.6

* Based on the observed presence of yellow bodies.

Infections occurred in 8 of the 13 strains (representing 6 species) of snails exposed to transmission by one or another of the 4 experimental methods. These included all strains of *Helisoma*, *Australorbis*, *Biomphalaria*, and *Bulinus*. Attempts to infect *Lymnaea palustris*, *Physa heterostropha*, and *Marisa cornuarietis* by means of inoculation or environmental contamination were not successful. Similarly, infections could not be established by inoculation in the two land snails, *Otala lactea* and *Helix aspersa*. It would appear, from our limited experience, that this mycobacterium shows some degree of host specificity in that infection occurred only in snails belonging to the family Planorbidae. Infection of snails by environmental contamination, or by the introduction of infected individuals, was exceedingly

TABLE 3

Summary of successful transmission experiments with the snail acid-fast bacillus

Species	Method of exposure	Controls infected	Exp. snails* infected	Day infection first noted
H. anceps	Environmental	0/20	11/20	15
H. trivolvis	Stab wound†	0/10	4/10	6
H. t. fallax (1)	Environmental	0/10	7/20	17
H. t. fallax (2)	Environmental	0/10	4/10	28
H. t. fallax (2)	Stab wound	0/5	3/5	10
A. glabratus (PR-1)‡	Infected snails	0/20	7/30	31
A. glabratus (PR-1)‡	Environmental	0/20	5/20	20
A. glabratus (PR-1)	Stab wound	0/5	2/5	4
A. glabratus (PR-1)‡	Inoculation	0/20	12/20	6
A. glabratus (M)	Environmental	0/10	5/10	14
A. glabratus (M)	Inoculation	0/10	8/10	5
B. truncatus	Environmental	0/10	2/20	25
B. pfeifferi	Environmental	0/10	1/10	43
B. pfeifferi	Stab wound	0/5	3/5	12
B. pfeifferi	Inoculation	0/10	4/10	5

* Snails which died during the course of the experiment and exhibited no gross evidence of infection were considered negative.

† In inoculation and stab wound infections, the formation of a lesion in a tissue other than the site of infection was required if the snail was to be considered infected.

‡ Represents data from duplicate experiments.

variable; several experimental groups were exposed for periods exceeding 90 days without discernible infection. Details of successful transmissions are given in Table 3.

Bacilli were found in tissue sections as early as the 14th day after exposure to environmental contamination, although yellow bodies were not grossly detectable until the 22nd to 30th day. Inoculation and stab wound infections produced grossly detectable lesions as early as 4 to 5 days later with the average at about 8 days. During the course of the experiments no differences could be detected between control and experimental snails in respect to growth, fecundity, or feeding behavior. Mortality was observed, but it was not excessive and could not be directly attributed to mycobacterial infection.

COMMENTS

We were primarily interested in the acid-fast bacillus as a possible agent for the biological control of schistosome-bearing snails. Disease produced by this organism in snails appears to be chronic rather than acute and neither extensive morbidity nor mortality occurs. Unless the virulence of the organism can be considerably increased, its value as an agent for biological control is questionable.

Recent studies have emphasized the association between certain atypical acid-fast bacilli and human clinical disease.[10–12] Many characteristics of the snail bacillus—morphology, chromogenicity, temperature tolerance, etc.—show similarities to those of atypical organisms described from man. It is possible that future work may demonstrate that the mycobacteria of snails and of other invertebrates must be considered in the epidemiology of human diseases resulting from infection with atypical acid-fast bacilli.

SUMMARY

A pathogenic acid-fast bacillus was isolated from the fresh-water snail *Helisoma anceps* and has been successfully maintained in pure culture. Techniques for the isolation and maintenance of this organism, as well as observations on its pathogenicity, are described. The results of experimental transmission of the organism to 13 strains of fresh-water snails, representing 6 species in 4 genera, are also presented.

ACKNOWLEDGMENTS

I wish to particularly acknowledge the aid, with respect to histologic techniques, given by my colleague Dr. C-T. Pan. It is likewise a pleasure to acknowledge Drs. Eli Chernin and Thomas Weller for their aid in the preparation of the original manuscript.

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PLATE I

FIG. 1. Camera lucida sketches showing malformations in the tentacles of snails, *Australorbis glabratus* (A–B) and *Helisoma anceps* (C–D), infected with the acid-fast bacillus. Note the enlargement of the tips of the tentacles and the accumulation of pigment. Yellow bodies (yb) are seen in specimens A and D. × 37.5.

FIG. 2. Longitudinal section through a portion of the anterior head region and tentacle of *H. anceps*. Yellow body lesions which were observed on gross examination are seen as tubercles "a," "b," and "c" at the base of the tentacle. Note the aplasia at the tip of the tentacle and the large pigment particles. Hemalum and eosin, × 65.

FIG. 3. Tubercle "a" (Figure 2) at higher magnification. Note fibroblasts which surround the tubercle and the inner mass of necrotic cells. Hemalum and eosin, × 360.

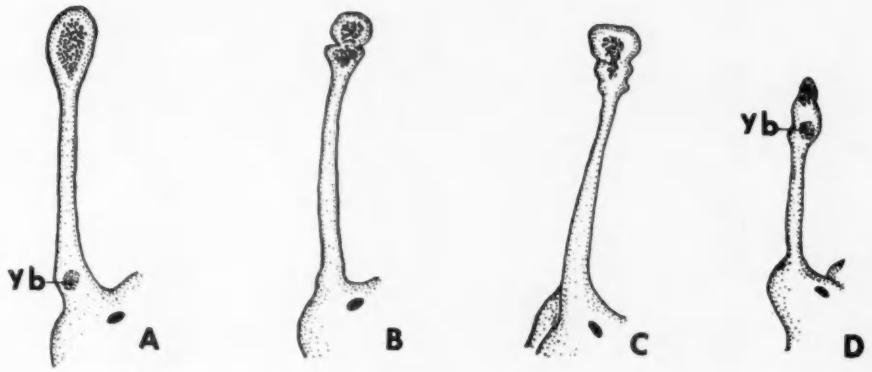

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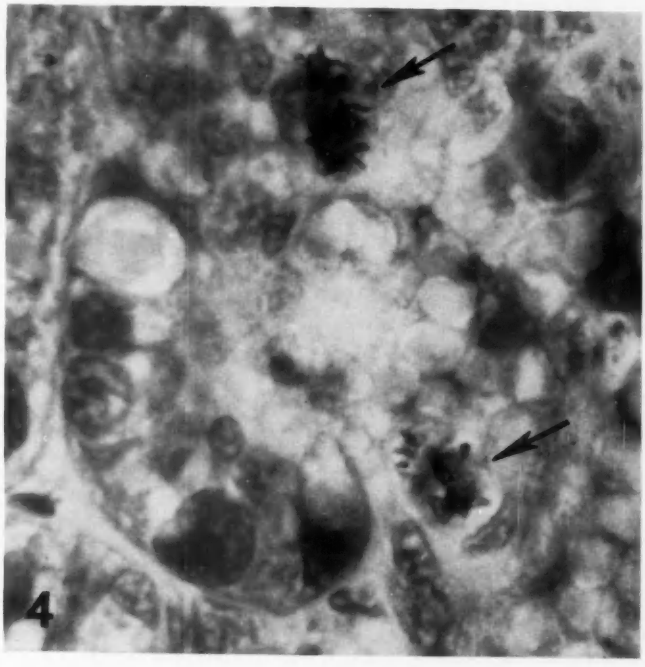
PLATE II

FIG. 4. Section through the digestive gland of *Helisoma anceps* showing individual amebocytes (arrows) infected with acid-fast bacilli. The snail was exposed to experimental infection (environmental contamination) 7 days prior to sectioning. Carbol fuchsin and Janus green, × 1,600.

FIG. 5. Section showing an aggregate of infected amebocytes and tissue cells in the connective tissue surrounding the digestive gland. The snail (*H. anceps*) was exposed to experimental infection (stab wound) 14 days prior to sectioning. Carbol fuchsin and acid hemalum, × 1,600.



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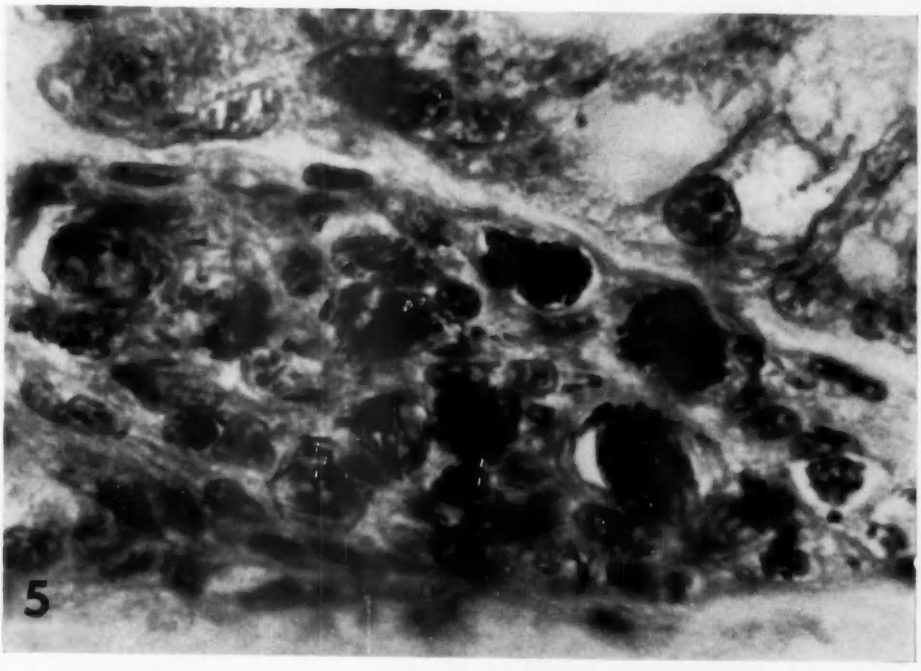

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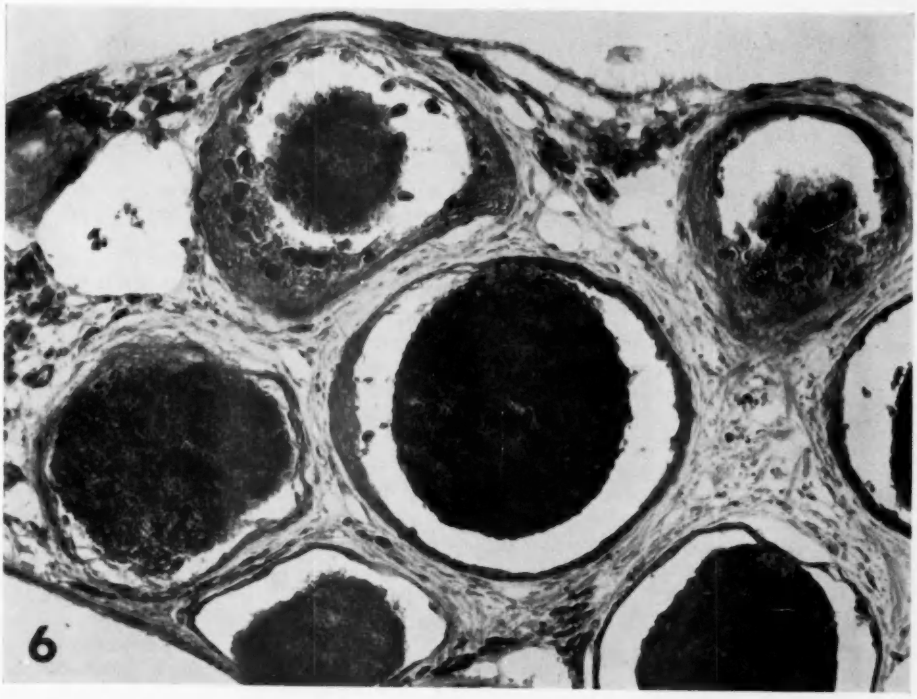
PLATE III

FIG. 6. Section of a biopsy taken from the mantle collar of *Helisoma anceps*. The snail became spontaneously infected 4 months prior to sectioning and the pathology is representative of the chronic stage of the disease. Note well defined cavernous tubercles filled with masses of acid-fast bacilli. Carbol fuchsin and acid hemalum, × 260.

FIG. 7. Longitudinal section through a spontaneously infected *H. anceps*. Multiple lesions are seen in the kidney, rectal ridge, and foot. Carbol fuchsin and acid hemalum, × 32.



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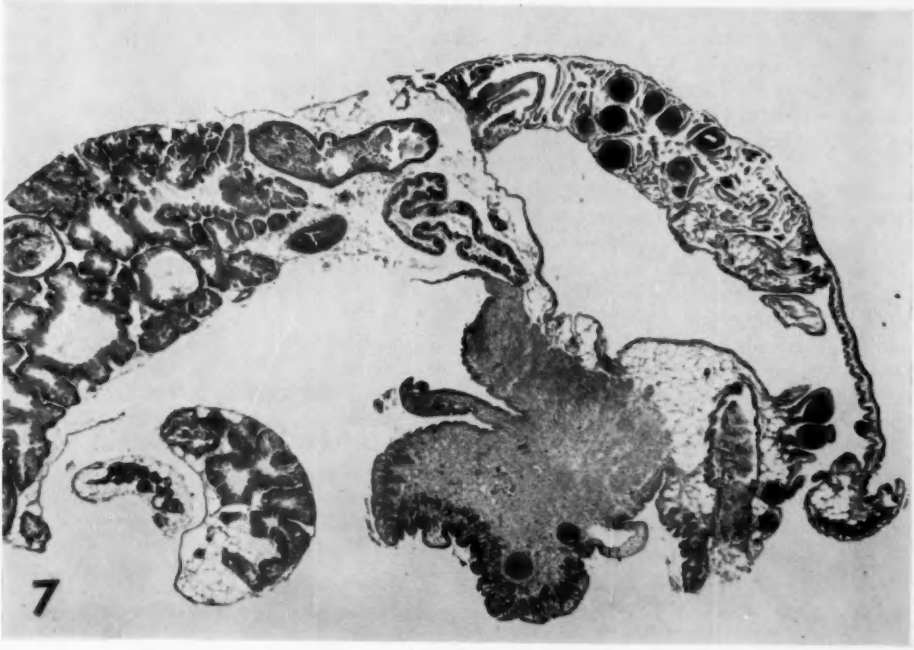

7

FIELD SURVEY OF THE HEALTH OF BACHIGA CHILDREN IN THE KAYONZA DISTRICT OF KIGEZI, UGANDA*

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Kigezi is the most western district of Uganda, bordering for the most part on the Congo and Ruanda-Urundi (Figure 1). It is mountainous and in many parts is becoming increasingly densely populated, especially around the main town of Kabale, where the population is about 600 per square mile. In consequence, the Bachiga, the Bantu tribe which makes up the majority (78.5%) of the local population, has laboriously had to carry terraced agriculture up even the steepest slopes (Figure 2).

The resultant pressure on the land is associated with considerable poverty; and the long, exhausting distances between villages and hillside fields or streams and other sources of water are a continuous strain on the women, who are responsible for all cultivation. This also means that, while young babies will be taken along on their mothers' backs, toddlers will be left behind and looked after, often rather haphazardly, by older siblings or elderly female relatives. Cow's milk and other sources of animal protein are rare; there are no fish ponds at the higher altitudes. In addition, there is a trend towards the cultivation of plantain (1% protein), as a "prestige food," and for beer rather than the more traditional millet (6% protein), which, anyway, requires prolonged grinding before eating as compared with the easily prepared *matoke* (steamed plantain), which only has to be peeled before cooking.

For these reasons, protein-calorie malnutrition in early childhood is considered to be common in the densely populated parts of Kigezi, so that different kwashiorkor syndromes are recognized by the Bachiga, depending upon the main features, as shown by the following names: *eyoshe* (child displaced by a subsequent pregnancy), *mutuku* (referring to the light colored hair and skin), and *embeho* ("coldness" referring to the edematous feet).

Kwashiorkor accounted for 23% of 995 admissions of children up to 3 years of age to Kabale Hospital in 1959; while 10.5% of 1 to 3-year-old children attending various dispensaries throughout Kigezi in 1957 were suffering from this syndrome.[1] In another investigation, 5.4% of infant dispensary attenders were recorded as having "marasmus."[2] These figures taken from hospitals and dispensary attendances do not, however, show the actual community incidence, which is unknown, and they may well give a somewhat more grave position than is really the case, in view of the large population draining into limited medical facilities.

The cold damp mountainous climate also affects the local disease pattern in children, so that the principal cause of admission to Kabale Hospital in the 1- to 3-year-old group in 1959 was respiratory tract infection (54%). Laboratory examinations of thick blood films taken from all age groups seen at this hospital in 1959 for parasites showed malaria to be relatively uncommon (14%), while stool examinations showed that ascariasis was widespread (42%) and ancylostomiasis rare ($< 1\%$).

As the population in much of Kigezi continues to rise and as family planning in these rural circumstances is, at best, a thing of the future, resettlement in as yet underpopulated parts of the region appears to be at least a possible temporary solution to the problem. The plains, especially around Kihihi, are the site of a planned government resettlement scheme, which has been made possible by a malaria eradication project being carried out in collaboration with the World Health Organization, although the sociological problems of a hill-dwelling people adapting to life in the plains will be considerable.

An alternative source of land is to be found as a result of the gradual opening up of the Impenetrable Forest (Figure 3) by its erosion from the edges by the clearings of peasant farmers. The *gombolola* (sub-county) of Kayonza,

* Community Child Health Studies in East Africa No. 1.

FIG. 1. Map showing Kayonza, Kigezi, in relation to Kabale and to the Impenetrable Forest. U = Uganda, K = Kenya, T = Tanganyika, R. U. = Ruanda-Urundi and E = equator.

lying on the edge of this densely forested region, has a population of about 9,600 (150 per square mile) which has doubled in the last 5 years, largely as a result of immigration. It is mostly hilly, with altitudes varying from 3,000 to 7,000 feet, coming down to the plains for a small area near Ntungamo.

Houses are widely scattered; there are no villages. Dwelling places are of mud and wattle, with thatched roofs, and with areas of cultivation surrounding them. Millet is stored in small thatched and woven containers situated between the huts. Fuel is easily available and water, from a stream, is usually nearby. The closeness of the forest means that constant baboon-watching has to be kept up by children who sit in little, specially made observation huts overlooking the hilly fields.

Basic crops are finger millet (*Eleusine coracana*), sorghum (*Sorghum vulgare*), maize, beans (*Phaseolus vulgaris*), peas, potatoes (both sweet and Irish), and, in more low-lying parts, plantains. Cattle and pigs play no significant part in the local diet; at the time of the survey there were only eleven cows in the whole *gombolola*. Chickens and goats are common, but the

FIG. 2. Intensely cultivated part of Kigezi near Kabale, showing terracing extending up steep slopes

FIG. 3. The Impenetrable Forest, near Kayonza, slowly being encroached upon by clearings of peasant farmers.

latter are not milked. Game probably plays only a minor role in the local dietary, although wild pig and various buck are hunted with spears, and birds and small animals are trapped. Bought food also is of little importance to the local peasant farmer and his family. In the whole *gombolola* there were eleven "shops" offering a very small range of items such as matches and kerosene, and of luxuries such as tea and sugar.

Minority groups are found in Kayonza, notably Banyuaruanda, Banyabatumbi and Batwa (pygmies). Although examined in the course of this study, they did not form a significant enough proportion to be included in the main body of results.

Health services for an area "at the end of the road" such as Kayonza are difficult to organize because of the lack of communications, scattered homesteads and the ever-present limitations of finance and trained staff. In fact, a dresser visits the *gombolola* headquarters weekly with a limited supply of drugs, and cases can be referred to the dispensary at Kanungu. Under these circumstances, most treatment and prevention of disease will be carried out by local methods, whether by the family or by the indigenous practitioner,[3] as was, indeed, verified by the patterns of medicinal scarification found very commonly on children's bodies.

Financially gainful occupations are, at present, few, except for the sale of produce or livestock, such as goats. However, the development of the timber industry and the discovery of mineral resources may alter matters, and attempts are being made to introduce tea as a homegrown cash crop for the peasant.

SURVEY METHODS

Aims

The survey was undertaken in December, 1959, and was aimed at examining the total child population aged between birth and 3 years in the district of Kayonza, in order (1) to assess on a community basis the prevalence of malnutrition (especially in the vulnerable 1- to 3-year-olds), intestinal helminthic infection, malaria and various other diseases in early childhood, and (2) to gauge the commonness of various conditions liable to become public health problems in any age group and, therefore,

FIG. 4. Temporary grass huts erected for survey

to make this region less suitable as an overflow area for the more crowded parts of Kigezi.

Organization

The child health survey was a by-product of a survey of the district undertaken 1 year after an initial mass treatment campaign for yaws with penicillin. A census of those attending the previous time, representing as far as possible the complete population, was available. The district chief and his subordinates were responsible for the attendance of people at prearranged collecting points at which temporary grass huts were erected (Figure 4). Family units, which could be large if polygamous (Figure 5), were examined for yaws by inspection and subsequently children up to 3 years of age were passed to the child health team for examination (Figure 6).

It was assessed that there was about a 93% reattendance, largely owing to the authority of, and explanation by, the chiefs, but also to the popularity of a yaws survey, with the possibility of an injection, to the general novelty of a medical inspection, especially with such mysteriously satisfying techniques as stethoscopy, and to the availability of a number of drugs for treating sick children.

Techniques

Four teams visited the area for a week each. Several meetings were held beforehand in order to coordinate techniques and to ensure agreement as to definition of the physical signs recorded. A "line of flow" was arranged in the examination hut and the following data were recorded on "Findex" punch cards:

1. *Social details.* Name, age, sex, and number of siblings alive and dead were recorded.

2. *Weight.* A simple basket scale was used, giving results to half a pound. Heights were not recorded in view of the great difficulty of carrying out this measurement with any accuracy in rural circumstances in frightened, struggling young children.

3. *Clinical examination.* This was entirely inspectional, using carefully predefined, clear-cut signs, except for auscultation for cardiac murmurs. The signs looked for are listed below: only positive findings were ticked on the appropriate number on the card.

FIG. 5. Better-to-do polygamous family waiting for examination

Kwashiorkor	Body ringworm
Incomplete kwashiorkor	Scalp ringworm
Nutritional marasmus	Impetigo
Nutritional dwarfing	Yaws
Clinical rickets	Jiggers
Congenital abnormality	Miliaria rubra
Charms	Leprosy
Hypochromotrichia	Molluscum contagiosum
Anemia	Follicular hyperkeratosis
Conjunctivitis	Pellagra rash
Corneal scars	Flaky paint rash
Bitôt's spots	Mosaic skin
Neck glands	Medicinal incisions
Ear discharge	Vaccination scar
Nasal discharge	Cardiac murmur
Angular stomatitis	Umbilical hernia
Caries	Palpable liver
Clinical scurvy	Palpable spleen
Scabies	Pretibial edema
Sores	

Definitions used for the four syndromes of severe protein-calorie malnutrition (first four in the list) and for hypochromotrichia, pretibial edema etc. have been given elsewhere.[4-6]

4. *Diet.* Approximate qualitative dietary histories were taken from mothers by a member of one team, a Luchiga speaking medical student (S. R. K. Sebikari), who also noted whether the

FIG. 6. Bachiga awaiting examination for yaws followed by child health survey.

child was being breast-fed or not at the time of inquiry.

5. *Laboratory tests.* A thick blood film was taken from all children and stained with Giemsa for malarial parasites, and a hemoglobin estimation (Sahli) was made on every fifth child. Stool samples were *taken* from each child by inserting a smooth-edged glass tube (about one-fourth inch in diameter by 3 inches long) into the anus. The small core of feces so removed was then pushed by a piece of wire into a tube containing a small quantity of 5% formol-saline, which preserved the ova and cysts, permitting examination weeks later.

RESULTS

Nutrition

Syndromes. The clinical nutritional status of the 258 infants and 585 preschool age children examined was surprisingly good. Of the main protein-calorie malnutrition syndromes, kwashiorkor (Figure 7) was seen in 2 (0.3%) and nutritional marasmus in 9 (1.5%) 1- to 3-year-olds. No infant had kwashiorkor and in only two (0.8%) was nutritional marasmus seen. No clinical rickets, infantile scurvy nor vitamin B deficiency syndromes were noted in either group.

FIG. 7. Kwashiorkor in 2-year-old child, showing apathy, light and sparse hair, edema and muscle wasting. He has been displaced from the mother's breast by the younger sibling. (Kwashiorkor was *rare* in Kayonza, being seen in only two children.)

TABLE 1

*Nutritional indices in Bachiga children**

	Total examined	Edema		Hypochromotrichia		Angular stomatitis	
		No. pos.	Index	No. pos.	Index	No. pos.	Index
Infants	258	0	0	38	14.7%	0	0
1- to 3-year-olds	585	2	0.3%	108	18.5%	2	0.7%

* Bitôt's spots, follicular hyperkeratosis and pellagra rash were not seen.

TABLE 2

Weights of 228 Bachiga children from a typical area, assessed by the Gómez classification

	Total examined	Gómez weight classification					
		1st degree		2nd degree		3rd degree	
		No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.
Infants	79	21	27	2	3	0	0
1- to 3-year-olds	149	57	38	17	11	0	0

Nutritional indicators. Nutritional indices, based on the use of various "indicators," also showed comparatively few abnormalities, compared with findings in most tropical regions (Table 1).

Gómez weight classification. Similarly, as judged by weight deviation below normal, expressed by the Gómez classification,[5] the nutritional status of the young children of this community was not markedly subnormal. Standards used were those for the children of another tribe, the Baganda, collected by Dr. R. F. A. Dean of the Infant Malnutrition Research Unit, Mulago Hospital; these may not be completely appropriate for the Bachiga.

The usual difficulties were experienced with regard to age estimation, so that results are included in Table 2 for children for whom approximate checks of age were made by local "time-marking" incidents.

Parasites

Malaria. There was but little evidence of malaria. The spleen rate was 0.8% for infants

TABLE 3

Results of stool examinations in Bachiga children

	No. of stools	*Ascaris*		*Trichuris*		Hook-worm		*Enterobius*		*Taenia*		*E. histolytica*		*Giardia*		Mixed infections	
		No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.
Infants	136	12	8.8	0	—	1	0.7	0	—	0	—	0	—	3	2.1	1	0.7
1- to 3-year-olds	220	131	59.5	10	4.5	4	1.9	2	1.0	3	2.9	4	1.9	23	8.9	26	11.8

TABLE 4

"Environmental" skin lesions in Bachiga children

	No. examined	Scabies		Sores		Body ringworm		Scalp ringworm		Impetigo		Jiggers		Molluscum contagiosum	
		No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.
Infants	258	23	8.9	14	5.4	4	1.6	1	0.4	5	1.9	0	0	0	0
1- to 3-year-olds	585	60	10.3	90	15.4	6	1.0	8	1.4	64	10.9	14/251	5.6	11/251	4.4

and 0.5% for 1- to 3-year-olds. Thick blood films examined from infants showed no positives. Among slides from 585 preschool age children, only 8 (1.6%) were positive, all with *Plasmodium falciparum*. The low incidence of malaria would seem to be related to the altitude of much of the area and to the malaria eradication scheme that was being carried out in the adjacent plains. These findings agree with those of previous investigators.[1, 2, 7]

Intestinal parasites. Results of 136 stool examinations in infants and 220 in 1- to 3-year-olds are shown in Table 3.

Analysis of results shows that, in main, little infestation appears to occur in the 1st year of life, but that, in the preschool age group, ascariasis is common (59.5%), giardiasis not infrequent (8.9%) and ancylostomiasis relatively unusual (1.9%). (Similar findings in relation to the relative prevalence of roundworm and hookworm infections have been made previously.)

Observations as to whether or not ova were present in large numbers in the stool were difficult to interpret owing to the somewhat variable amount of feces obtained by the anal tube technique. Similarly, the rather low prevalence of *Entamoeba histolytica* may be partly due to the use of formol-saline as a preservative.

Onchocerciasis. Although occasionally older children and adults were seen with characteristic onchocerca nodules and/or dermatosis, no case was recognized in younger children aged up to 3 years.

Skin Diseases

Various clinically diagnosable infective lesions, mainly the result of environmental factors, such as dirt, overcrowding and lack of abundant water, were common (Table 4).

The most unexpected finding was that molluscum contagiosum was so common (being present in 4.4% of 251 children) that it was recognized locally as an entity and referred to as *empengeri* (meaning "grain of boiled sorghum"), an excellent descriptive term. Leprosy appeared to be uncommon in these young children, at least as an easily recognizable clinical entity, as no definite cases were noted. Lesions clinically suggestive of active yaws were relatively uncommon and, among children aged up to 16 years examined at the same time by the Yaws Control Team, only 1.8% were positive. (The striking improvement following the initial mass single-dose penicillin campaign in 1958 is indicated by the fact that at that time 20.6% of children of this age group were found to be positive.)

"Prickly heat" (miliaria rubra), as might have been expected, was rare in older children in this cool, hilly region. It was noted only in infants (7.0%) and apparently was due to the

FIG. 8. Medicinal brand marks on abdomen, indicating past *ekintu*, with main symptom diarrhea.

hot "micro-climate" resulting from contact with the mother's back.

Superficial Infection

Apart from the skin lesions noted above, what may be termed evidence of "superficial infection" was found in infants and in 1- to 3-year-olds, respectively, as follows: conjunctivitis, 16.3% and 14.4%; nasal discharge, 14.4% and 42.6%; aural discharge, 0% and 0.9%; and neck glands (mostly posterior triangle), 1.2% and 2.7%.

Skin Scarification

Vaccination. Evidence of vaccination against smallpox, as judged by a typical scar, was very uncommon, being found in none of the infants and in only five 1- to 3-year-olds (0.9%).

Medicinal incisions. As opposed to charms which were infrequently seen, linear medicinal incisions or circular branding marks were found in 7.0% of infants and 28.9% of the older group (Figure 8). Apart from this indication of dependency on indigenous medical methods, analysis of the scar pattern was undertaken after inquiry into the symptom complexes of the culturally defined diseases for which each pattern was made (Table 5).

Infants with medicinal scar patterns showed the following distribution: chest 57.2%, abdomen 21.4% and forehead 21.4%. Among the preschool age group, the pattern distribution was chest 65.2%, abdomen 13.0%, lower back 11.5%, forehead 7.2% and eyelids 5.8%.

Childhood Mortality

While at present it is not possible to calculate infant and preschool age mortality rates in Kayonza, the "sibling mortality"—that is, the percentage of nonsurviving siblings of children who attended—gives an index of mortality in childhood. Results of questioning 132 mothers showed the sibling mortality to be 18%, a relatively low figure by tropical standards, and

TABLE 5

Sites and possible significance of scar patterns found in Bachiga children

Site	Disease name (Bachiga)	Symptoms	Possible scientific equivalent
Forehead	*Omutwe*	Severe headache	?
Eyelids	*Amaisho*	Discharge from eyes	Conjunctivitis
Chest	*Omushonga*	Cough, chest pain, difficulty in breathing	Pneumonia
Abdomen	*Ekintu**	Diarrhea (with frothy, yellowish-green stools)	Gastroenteritis
Low back (sacral region)	*Omugongo*	Diarrhea	Gastroenteritis

* The word *ekintu* means "thing," a culturally acceptable way of avoiding the name of a feared disease, as with the avoidance of the word "death" in the English language.

in surprisingly close agreement with Gongora and McFie's estimate of 17.2%,[3] but suggesting that one child out of every five born dies in childhood.

A death register is kept in the *gombolola* headquarters and, although probably only a fraction of deaths occurring are, in fact, registered, an analysis was carried out on the 444 records made between January, 1952, and October, 1959. This showed that approximately 51% of all the deaths recorded had been in children 5 years old or younger, and, among these children, the "causes" of death were listed under certain general descriptive titles, which could be grouped as follows: "cough" or "pneumonia" 81%, "fever" 8%, kwashiorkor 4%, whooping cough 3%, miscellaneous 4%. Of the children in the "cough" or "pneumonia" group, 44% were infants, that is below 1 year of age. It is notable that diarrhea is not listed among the causes of death.

Miscellaneous

Anemia. Results showed that in approximately 3% the hemoglobin was below 7.5 grams per 100 ml and in 20% it was over 11 grams per 100 ml. In the majority (77%) it fell between 7.5 and 11 grams. These findings must be correlated with the fact that both ancylostomiasis and malaria are uncommon in these children, while sickle cell anemia is probably not frequent as the sickle cell trait is found in only about 4%.

Palpable liver. Probably abnormally palpable livers were found in 2.3% of infants and 3.9% of 1- to 3-year-olds. This is a very low incidence, presumably related to the uncommonness of malaria in the region and the apparently moderate level of nutrition.

Caries. In the preschool age group, caries of one or more teeth was found in only 22 children (3.8%). The absence of sugar and of overrefined flour from their diet may be responsible, while it may be noted that millet is a good source of both calcium and fluorine.

Congenital abnormalities. Clinically apparent anomalies were found in 14 infants (5.4%) and in 27 of the older group (4.6%). For the most part these were minor. For example, in 245 children examined by one team the two minor and harmless abnormalities, preauricular sinus (69.2%) and supernumerary digits (15.4%) were commonest, together with occasional other anomalies, including strabismus, microcephaly and so on.

Cardiac murmurs. Systolic murmurs were found in 0.2% of infants and 0.9% of preschool children.

Umbilical hernia. This condition, which has been shown to be due to a physiological variation of neonatal umbilical structure, was present in 10.0% of infants and 5.8% of the older group. This is a much lower incidence than in many other parts of Africa, including Western Nigeria.[8]

DISCUSSION

In East Africa, as in many tropical regions, seven groups of conditions constitute the bulk of disease in early childhood—protein-calorie malnutrition, infectious diarrhea (gastroenteritis), intestinal helminth infections (especially ascariasis and ancylostomiasis), tuberculosis, anemia (due to various factors including sickle cell disease, hookworm disease, malaria), bronchopneumonia and malaria. With the data available, the prevalence of many of these entities among children in Kayonza could be assessed. Protein-calorie malnutrition, often the main disease of tropical preschool children, appeared to be uncommon, at least in its more gross forms, in the young children of this region. For example, the edema index in preschool children in the West Indian island of Haiti was 7%,[9] as opposed to only 0.3% in the present study. In addition, although similar community studies have not been undertaken, malnutrition seems much less common in Kayonza than in the country around the main town of Kabale itself, so that the present survey must be considered as probably definitely atypical of Kigezi as a whole.

Possible reasons for this are several. As compared with the country around Kabale, there is as yet no population pressure and distances from houses to fields or water are small so that mothers do not have to exhaust themselves with long journeys and, at the same time, do not have to leave young children so long at home to be cared for by siblings.

It would seem that the methods of infant feeding employed in Kayonza must be partly responsible; there is also a very low incidence of malaria and of hookworm disease, two diseases which are important conditioning factors in other parts of the country.

Pattern of child feeding. Qualitative data were obtained from mothers seen by one team by

TABLE 6

Principal items of diet taken by 61 breast-fed Bachiga infants during the 1st year of life

Age group (months)	No. in group	Additional food		Millet gruel		Plantain		Vegetable marrow		Maize porridge		Irish potato		Beans	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
0–3	28	2	7.1	2	7.1	0	0	1	3.6	0	0	0	0	0	0
4–6	19	10	52.6	9	42.1	7	36.8	2	10.5	1	5.3	1	5.3	0	0
7–9	10	9	90	8	80	9	90	0	0	0	0	2	20	1	1
10–12	4	4	100	4	100	4	100	2	50	0	0	1	25	2	50

TABLE 7

Principal items of diet taken by 65 Bachiga children in the 2nd year of life

Breast feeding		Additional foods		Plantain		Millet gruel		Beans		Sweet potato		Millet meal	
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
65	83.1	65	100	61	93.8	57	87.7	42	64.3	39	60.0	26	40

using a questionnaire as to present diet. The diet fell into a clear-cut pattern (Tables 6 and 7). Breast feeding was universal throughout infancy, with the addition of small amounts of semi-solids by some parents even in the first 6 months of life and by all of them in the second semester. The two principal foods added were millet (in the form of the gruel *obushera*, prepared by soaking either finger millet or sorghum mixed with ash for 24 hours to permit germination, after which it is dried and ground to a flour) and steamed plantain (*matoke*). Occasionally, beans (*Phaseolus vulgaris*) were given to children later in the 1st years.

Breast feeding was continued into the 2nd year for 83.4% of the children. All children were also receiving a mixed diet, the main items of which were, in order of commonness, plantain, millet gruel, beans, sweet potato and millet meal (*buro*). In a number of children, breast feeding was still being carried on during the 3rd year of life.

"Family spacing techniques," such as practiced in many parts of Africa, do not seem to be important, save for polygamy which is usual. Thus, there is no ban on sexual intercourse during lactation. Cessation of breast feeding is aided by painting the breasts with bitter herbs. Foster mothers, usually lactating relatives, are employed if a mother dies in childbirth, and the phenomenon of "induced lactation" is well known, even among grandmothers.[10]

Animal protein foods, such as cow's or goat's milk, eggs, fish, meat or shop-bought dried milks, played an insignificant part, any of these items at best being only used in small quantities at intervals of at least a week. Apart from scarcity of these foods and their value as "cash crops," certain food prejudices may be relevant. Thus, goat's milk is not drunk not only because it does not form part of the local traditional dietary but also because it is believed to give rise to scabies. However, in a rapid survey of this type, it was not possible to investigate in detail local nutritional "cultural blocks."[11]

Bottle feeding with its attendant hazards of starvation and infectious diarrhea, as stressed by Welbourn[12] and Jelliffe *et al.*,[6] has not, as yet, reached this region, nor has the often parallel falling-off of breast feeding noted in many tropical countries.[13]

It would seem then that protein-calorie malnutrition syndromes, such as kwashiorkor and nutritional marasmus, are avoided at least in this less crowded rural part of Kigezi by the excellent "transitional" diet resulting from breast feeding prolonged into the 2nd year in most instances and from mixed feeding with the two most valuable local sources of vegetable protein, millet and beans, introduced in the second 6 months of life and subsequently.

Of the other important diseases of tropical childhood, very severe anemia, malaria and ancylostomiasis were not frequent, although ascariasis was common.

The incidence of infectious diarrhea is im-

possible to determine in a community-wide study, but admission figures for Kabale Hospital do not suggest that it is an important problem. The prevalence of tuberculosis could not be measured, as it would have been impossible to have children reattend after 2 to 3 days in order to observe the results of tuberculin tests.

By contrast, there seems little doubt that severe respiratory infections possibly including pulmonary ascariasis, jointly labeled as "bronchopneumonia," are the topmost cause of severe illness and death in this region. Evidence for this can be obtained from admission figures to Kabale Hospital, by the medicinal scar patterns on children examined and by the approximate records available in the death register.

SUMMARY

A field survey of child health was carried out in the rural district (*gombolola*) of Kayonza in Kigezi, West Uganda. The total population of young children was examined (258 infants, 585 1- to 3-year-olds). Results showed very little evidence of protein-calorie malnutrition (including kwashiorkor); the edema index in 1- to 3-year-olds was only 0.3%. While minor degrees were frequent, severe anemia was found in only 3% in the preschool age group. Malaria was uncommon, as judged by a spleen rate of 0.5% and a parasite rate of 1.6%; stool examinations showed *Ascaris* in 59.5% and hookworm in only 1.9%. Skin disease due to "environmental" factors was common. Indirect evidence, including admissions to Kabale Hospital, medicinal scar patterns in children examined and records in the death register, shows that severe respiratory infection is the top cause of morbidity and mortality.

The relative absence of protein-calorie malnutrition is probably due to a variety of factors including the excellent transitional diet (prolonged breast feeding, millet, beans) and to the lack of land pressure. A much worse situation would be expected in the overcrowded parts of Kigezi, especially around Kabale.

ACKNOWLEDGMENTS

The teams were greatly assisted in the field by the following medical students: S. R. K. Sebikari and J. K. Kyewalabye, Makerere Medical School; and B. A. Wharton and M. Kay, Nuffield Tropical Medicine Scholars, Medical School, University of Birmingham, England.

Thanks are due to Mr. Stephen Kalemire, Gombolola Chief, and his colleagues for the large scale organization needed; to the Yaws Team for valued assistance; and to Dr. R. F. A. Dean, Infant Malnutrition Research Unit, Kampala, for advice and helpful criticism.

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CORRESPONDENCE

Dallas, Texas, January 30, 1961

To the Editor.

Sir,

Dr. Donald L. Augustine has kindly called our attention to a reference which we inadvertently overlooked in the preparation of our manuscript "Additional records of *Dipylidium caninum* infections in children in the United States with observations on treatment" (*Am. J. Trop. Med. & Hyg.*, **9**: 604–605). The reference is that of Stuart, H. C., and Augustine, D. L., 1928, *Dipylidium caninum* infection in an infant 6 months of age, *Am. J. Dis. Children*, **36**: 523–525. This additional report makes a total of 14 such cases recorded from the United States instead of 13 as reported in our paper.

Sincerely yours,

DONALD V. MOORE
University of Texas
Southwestern Medical School
Dallas
FRANK H. CONNELL
University of Texas
Postgraduate School of Medicine
Baylor University School of Medicine
Houston

BOOKS RECEIVED

Handbuch der Urologie, C. E. Alken, Homburg (Saar), V. W. Dix, London, H. M. Weyrauch, San Francisco, and E. Wildbolz, Bern, Editors. Volume III, *Symptomatologie und Untersuchung von Blut, Harn und Genitalsekreten*, by Karl Hinsberg, Düsseldorf, Josef Kimmig, Hamburg, Johannes Meyer-Rohn, Hamburg, R.H.O.B. Robinson, London, Carl Schirren, Hamburg, and Renate Wehrmann, Hamburg. 307 + xv pages, illustrated (61 figures). Berlin, Göttingen, and Heidelberg, Springer-Verlag, 1960. DM 118.—. (With series subscription, DM 94.40.)

Handbuch der Urologie, C. E. Alken, Homburg (Saar), V. W. Dix, London, H. M. Weyrauch, San Francisco, and E. Wildbolz, Bern, Editors. Volume XII, *Funktionelle Störungen*, by Alfred Auerback, San Francisco, Carl E. Burkland, Sacramento, Gustav-Wilhelm Parade, Neustadt/Weinstrasse, J. Cosbie Ross, Liverpool, Donald R. Smith, San Francisco, and Walter W. Williams, Springfield. 312 + xiv pages, illustrated (74 figures). Berlin, Göttingen, and Heidelberg, Springer-Verlag, 1960. DM 98.—.(With series subscription, DM 78.40.)

You Can Prevent Illness, by Edward R. Pinckney, M.D. 157 pages. Philadelphia and New York, J. B. Lippincott Company, 1960. $3.50.

Theoretical and Practical Problems of Medicine and Biology in Experiments on Monkeys, I. A. Utkin, Director of the Institute of Experimental Pathology and Therapy of the U.S.S.R. Academy of Medical Sciences, Editor; translated from the Russian by Ruth Schachter, M.Sc. 276 + vii pages, illustrated. New York, Oxford, London, and Paris, Pergamon Press, 1960. $9.00.

Fortschritte der Geburtshilfe und Gynäkologie, A. Reist, Zürich, Editor. Volume 11, *Zum Problem der Toxoplasmose*. "Die Laboratoriumsdiagnostik der Toxoplasmose," by W. Roth, Basel; and "The Importance of Latent Maternal Infection with Toxoplasma in Obstetrics," by Josef A. Čech and Otto Jírovec, Prague. 90 + iv pages, paper covers. Basel (Schweiz) and New York, S. Karger, 1961. Sfr. 17.70.

Bibliography of Echinococcosis with Selected Abstracts, by Leland E. Powers, M.D., and Charles W. Churchill, Ph.D. 209 pages, paper covers. Beirut, American University of Beirut, 1960.

Pioneer Microbiologists of America, by Paul F. Clark, Emeritus Professor of Medical Microbiology, The University of Wisconsin. 369 + xiv pages, illustrated. Madison, The University of Wisconsin, 1961. $6.00.

Annual Review of Entomology, Edward A. Steinhaus, University of California, and Ray F. Smith, University of California, Editors. Volume 6, 470 + viii pages. Palo Alto, California, Annual Reviews, Inc. in co-operation with the Entomological Society of America, 1961. $7.00.

The Infectious Diseases of Domestic Animals with Special Reference to Etiology, Diagnosis, and Biologic Therapy, by William Arthur

Hagan, D.V.M., D.Sc., Professor of Veterinary Bacteriology Emeritus, New York State Veterinary College, Cornell University, Director, National Animal Disease Laboratory, U. S. Department of Agriculture, Ames, Iowa, and Dorsey William Bruner, B.S., D.V.M., Ph.D., Professor of Bacteriology, New York State Veterinary College, Cornell University. 4th edition, 1033 + xviii pages, illustrated. Ithaca, New York, Comstock Publishing Associates, 1961. $11.50.

The Rickettsial Diseases, by P. F. Zdrodovskii and H. M. Golinevich; translated from the Russian by B. Haigh, M.A., M.B., B.Chir., Late Physician to H.B.M. Embassy, Moscow, U.S.S.R. 629 + xii pages, illustrated. New York, Oxford, London, and Paris, Pergamon Press, 1960. $17.50.

BOOK REVIEWS

Human Toxoplasmosis. Proceedings of the Conference on Clinical Aspects and Diagnostic Problems of Toxoplasmosis in Paediatrics at The VIII International Congress of Paediatrics, Copenhagen, 1956, Revised and Edited 1959, J. CHR. SIIM, M.D., Director, The Toxoplasmosis Department, State Serum Institute, Copenhagen, Denmark, Editor. 220 pages, illustrated. Baltimore, The Williams & Wilkins Co., 1960. $12.50.

Just as toxoplasmosis has been a topic of particular interest at the meetings of the American Society of Tropical Medicine and Hygiene so has it been at various pediatric gatherings. During The VIII International Congress of Paediatrics in Copenhagen in July, 1956, a special conference was devoted to the "Clinical Aspects and Diagnostic Problems of Toxoplasmosis in Paediatrics." This meeting brought together specialists engaged in studies on toxoplasmosis in an attempt to organize in a clear-cut fashion the present knowledge of toxoplasmosis.

This small book is a compilation of papers on toxoplasmosis delivered at the special conference of the Congress in 1956, but has been revised in 1959. It thus provides relatively up-to-date information by a list of participants which includes some of the world's outstanding authorities on this infection. Following introductory remarks by Dr. Albert Sabin in which emphasis is placed on the principal problems of toxoplasmosis to which we require solutions there follow the reports of the eighteen other participants. Their contributions are grouped under the following headings: I. Congenital Toxoplasmosis, II. Acquired Toxoplasmosis, III. Treatment, IV. Ophthalmological Aspects, V. Epidemiology, and VI. Laboratory Diagnosis. Since the various articles represent the independent papers of the authors there is considerable duplication of content and the material is not as well organized as that of a text. Much space is devoted to case reports by several authors. These are of interest but contain information which is, in general, neither new nor unusual. There is one review of each major aspect of toxoplasmosis excepting laboratory diagnosis. These articles are the most valuable in the book. They include chapters by H. F. Eichenwald on congenital toxoplasmosis; J. C. Siim on acquired toxoplasmosis; D. E. Eyles on treatment; J. K. Beverly on ocular manifestations; and H. A. Feldman on epidemiology. These chapters present in well organized style a thorough review of each topic discussed and, even if published without the remaining articles, would together make a reference work of great value. The section on laboratory diagnosis is divided evenly among four contributors and stands as a better unit than any of the other sections, each article complementing the others.

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Handbook of Microbiology, by MORRIS B. JACOBS, Ph.D., Associate Professor of Occupational Medicine, School of Public Health and Administrative Medicine, Columbia University, and MAURICE J. GERSTEIN, B.S., M.S., Department of Biology, William H. Maxwell Vocational High School, Brooklyn, New York. 322 + x pages. Princeton, New Jersey, D. Van Nostrand Company, Inc., 1960. $8.50.

"Ready access to the data of microbiology is provided in this Handbook." With this objective in mind the authors have compiled an impressive mass of information and arranged it in convenient tabular form.

Table 1 contains characteristics of approximately 150 species of bacteria and fungi of medical and industrial importance. The organisms are listed alphabetically by genus and the table includes data on taxonomy, microscopic and cultural characteristics, biochemical reactions, and metabolic products. Industrial uses of the organism are included when applicable.

Valuable information is also provided in the

tables on temperature conversions; saturated steam temperatures; most probable numbers, preparation and range of hydrogen-ion indicators; preparation of test dilutions; antigenic formulae of the *Salmonella* and *Shigella*; determination of phenol coefficients; and species differentiation within many important genera.

In some instances, however, the book falls short of obviating the necessity for reference to other sources of information. While the table on antibiotics provides an extensive catalogue of products, descriptions of chemical and pharmacologic properties and spectrum of activity against microorganisms are often inadequate.

The table on stains and staining methods fails to provide sufficient information on both the preparation of stains and their application, thus severely limiting the usefulness of these data.

The tables on toxins, antitoxins, and antisera might well be expanded to contain information concerning protocol for immunization, commercial sources, and similar data of interest to immunologists.

The table on culture media and methods lists a wide variety of media but in many instances reference to other sources of information would be necessary for their preparation. The data on methods are generally inadequate in scope and detail.

In spite of its several weak areas, this handbook should prove useful to workers engaged in the medical, public health, and industrial aspects of microbiology. The data are arranged in a convenient and interesting manner; the book provides a unique and stimulating approach to handbook design. Expansion of the tables which are presently inadequate will undoubtedly enhance the usefulness of this handbook and lead to wider acceptance.

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Wärmestauung und Schwülekrankheiten. Entstehung, Verlauf, Behandlung und Verhütung für Ärzte und Hygienetechniker, by J. Grober, Professor an der Universität Jena. 200 + viii pages. Stuttgart, Gustav Fischer Verlag, 1960. DM 28.—.

In this book the author gives an objective account of the various factors involved in the production of the subjective sensation of sultriness and swelter. This difficult task is achieved by means of a thorough analysis of the meteorological components of macroclimate and microclimate and the physiological responses they elicit in man and animals. The term "Schwülekrankheiten" (diseases of swelter) is introduced, comprising all known disorders caused by heat, without separating them into distinct entities but considering them as different degrees of pathological response to varying atmospheric conditions. The disregard of the later literature on pathogenesis of disorders caused by heat, especially the recent work on the role of the endocrine system, is regretfully noted. The book contains many interesting personal observations on man and animals under the stress of heat and swelter and offers physiopathological hypotheses for the origin of these conditions, pointing out the need for experimental confirmation. There are well written chapters on balneological uses of heat and vapor, prophylactic-hygienic considerations of much practical value, and a comprehensive chapter on the treatment of chronic, light and severe "Schwülekrankheiten," all of which make this book very valuable to the practicing physician, the hygienist and the balneologist.

Victor Villarejos
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Atlas of Medical Mycology, by Emma Sadler Moss, B.S., B.M., M.D., Director, Department of Pathology, Charity Hospital of Louisiana at New Orleans, Clinical Professor of Pathology, Louisiana State University School of Medicine, and Albert Louis McQuown, B.S., B.M., M.D., Director, The Pathology Laboratory, Baton Rouge, Louisiana, Clinical Associate Professor of Pathology, Louisiana State University School of Medicine, Visiting Pathologist, Charity Hospital of Louisiana at New Orleans. 2nd edition, 335 + xi pages, illustrated. Baltimore, Williams & Wilkins Co., 1960. $11.00.

The growing importance of medical mycology is well demonstrated by the fact that the first edition of this book was twice reprinted within an interval of three years, and the second edition appeared seven years after the first edition. Not only have more chapters been added, but the format has also been enlarged.

The book is introduced by a glossary which is particularly useful for the uninitiated. However, there seems to be a slight departure from the generally accepted fact that the "crateriform" group is represented only by one species, *Trichophyton tonsurans*. *T. sulfureum* and *T. sabouraudi* thus should be considered cultural variations of that species. It also appears well established that *T. megnini* is a synonym of *T. rosaceum*, a species with well defined cultural and metabolic characteristics, and that neither should be considered as a synonym of *T. rubrum*. The recognition of *Geo-*

trichum and *Mucor* as pathogens is timely because the pathogenic role of these two fungi has been sadly neglected in the past. The laboratory part is presented in a simple and practical manner so as to meet the needs of not only the advanced, but also the beginner. The book is well produced, amply and clearly illustrated with numerous color plates. The bibliography is short, but adequate. It is altogether a well written book and is recommended for those who are interested in a concise and practical treatise of mycology.

FREDERICK REISS
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NEW YORK, NEW YORK

Infectious Diseases of Children, by SAUL KRUGMAN, M.D., Professor and Chairman, Department of Pediatrics, New York University School of Medicine, New York, N. Y., Director, Pediatric Service, Bellevue Hospital Center, New York, N. Y., Director, Pediatric Service, University Hospital, New York, N. Y., and ROBERT WARD, M.D., Professor and Head, Department of Pediatrics, University of Southern California School of Medicine, Los Angeles, Calif., Physician-in-Chief, Childrens Hospital, Los Angeles, Calif. 2nd edition, 398 pages, illustrated (55 illustrations and 7 color plates). St. Louis, The C. V. Mosby Company, 1960. $13.00.

The fact that a second edition of *Infectious Diseases of Children* by Krugman and Ward has appeared within two years of the first testifies to its popularity as well as to the rapidity with which new information is becoming available on the subject of infectious diseases.

The book should be of interest to all who have occasion to treat children with acute infections. The chapters on recently elucidated diseases, such as the adenoviral infections, the enteroviral infections, viral hepatitis (infectious hepatitis, serum hepatitis) and acute respiratory infections in general, are particularly welcome and outstandingly good, since the authors of the book have themselves contributed notably to our knowledge in these fields. Excellent also are the chapters on some of the "old favorites" such as chickenpox, measles, mumps, and streptococcal infections including scarlet fever. They are replete with simple diagrammatic charts and illustrations useful to the medical student, as well as results and discussion of newer investigations. The chapters on rabies and herpes virus infections are very complete, while the discussion of such subjects as acute bacterial meningitis, *Salmonella* infections, and *Shigella* infections is adequate. The authors are to be commended on including, under smallpox and vaccinia, Leon Schlossberg's fine life-like colored drawings of various types and stages of reaction to smallpox vaccination. The schematic representations in the chapter on diagnosis of acute exanthematous diseases will be appreciated by students and younger clinicians.

On the other hand, many readers will find the recommendations for the use of antimicrobial drugs confusing, listed as they are under various diseases; *e.g.*, penicillin is discussed in the chapter on measles, again under acute bacterial meningitis, staphylococcal infections, etc., with considerable variation in the suggested dosage and in the warnings concerning untoward reactions to the drug. This is particularly true in the case of chloramphenicol, which is recommended in the chapters on acute laryngitis and laryngotracheobronchitis, measles, acute bacterial meningitis, rickettsial infections, *Salmonella* infections, and staphylococcal infections; yet in only two of these six chapters is there mention of the serious toxic effects of the drug in young infants. A comprehensive discussion with recommendations and references collected in a single chapter on antimicrobial drugs might have been preferable, especially since the various drugs are not listed in the subject index.

While each of the thirty-one chapters concludes with an excellent list of references, both classic and recent, the indexing leaves a great deal to be desired. One of many examples of inadequate cross-indexing occurs in the chapter on toxoplasmosis, where a sentence points out the resemblance between this disease and cytomegalic inclusion disease, without, however, referring to any particular author; a reference listed at the end of the chapter is that of McElfresh and Arey on generalized cytomegalic inclusion disease. Each of these authors is listed in the author index but cytomegalic inclusion disease is nowhere listed in the subject index.

In the discussion of diphtheria one misses any reference to erythromycin, and, under tetanus, some reference to the disease as it is seen in the newborn, and to the use of chlorpromazine in management of both adults and infants; also, under *Shigella* infections, a sketchy discussion of the use of parenteral fluids might have been compensated for by inclusion of a few excellent references on the subject. Tuberculosis, still the most prevalent serious infectious disease in the world, is barely mentioned in passing. With the single exception of a good chapter on toxoplasmosis, protozoan, spirochetal, fungal and parasitic infections are not mentioned at all!

Notwithstanding these shortcomings, Krugman and Ward, in its second edition, presents the most satisfactory discussion of the infectious diseases of children as they are seen in temperate climates

and prosperous countries. For those who treat children in other parts of the world, especially those for whom current medical journals are not always readily available, the excellent discussion of very recent additions to our knowledge of viral infections in children may well compensate for the lack of information on problems of paramount importance to them.

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NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene—the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

Papers are ordinarily published in the order of reception, which entails a certain delay in their appearance because of the contractual limitation on the number of journal pages available for each volume. Accepted articles which raise no question of scientific priority but for which immediate publication seems important will be inserted in the next available issue of the Journal if the author or sponsoring institution will pay the cost, estimate of which may be obtained from the Editor. The insertion of such articles does not affect publication of other manuscripts awaiting their turn, since the inserted articles constitute additional pages in the volume without additional cost to the subscriber.

Publication will be facilitated if the following suggestions are followed:

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4. *Illustrations* should be protected with cardboard to avoid creasing in the mails; illustrations larger than 8½ x 11 inches should be accompanied by smaller photographs. Only glossy prints can be used. Line drawings and graphs should be made with India ink and submitted on white or blue tracing paper; they should be legible after reduction to one column. Colored illustrations can be reproduced only at the expense of the author.

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8. *References* should be cited by consecutive numbers in the text, and so numbered and listed in the list of references at the end of the article, without regard to alphabetical arrangement. References should be written as follows:

> 1. Reed, L. J., and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**: 493–497.

9. *Proprietary names* of drugs or chemicals registered by commercial pharmaceutical firms may not be used in the text if there is a generic name available in the U. S. Pharmacopoeia, New and Nonofficial Remedies of the A. M. A., or standard medical dictionaries. In certain cases where the generic name is relatively unknown, the registered name may follow it in parentheses in the title, at the first citation in the text, and in the summary.

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